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In order to evaluate the efficacy of herbal drugs, there need to be ways to qualitatively and quantitatively determine the constituents and their concentrations in various products. *Spilanthes* is a popular herbal extract which is used traditionally to treat stammering, stomatistis, and throat complaints. *Spilanthes* is commonly referred to as the "toothache plant," a name that refers to the analgesic action of its alkylamide constituents. A high performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS) method was developed to evaluate the quantity, identity, and stability of the alkylamides in ethanolic extracts of *Spilanthes acmella*. The developed method was then subjected to method validation according to guidelines given by the International Committee of Harmonisation (ICH) section Q2 (R1). The linear dynamic range for the method was determined to be 0.45 μM – 450 μM. The method detection limit and quantitation limit were estimated at 0.27 μM and 0.45 μM, respectively. The method precision was evaluated on two levels: repeatability and intermediate precision and was 6% or less. The method proves valuable for the quantitation and identification of the alkylamide spilanthol in *S. acmella.* Eight additional alkylamides have been tentatively identified (based on molecular weight and fragmentation patterns) in a 75% ethanol extract of *S. acmella*. The structure of the most abundant of these alkylamides, (2E,6Z,8E)-*N*-isobutyl-2,6,8-decatrienamide, spilanthol,

was confirmed by NMR. The spilanthol concentration in the extract shows no significant degradation during storage over six and a half months at room temperature, -20°C, and -80°C. *Spilanthes* extract and isolated spilanthol showed no antibacterial activity against *Staphylococcus aureus*.

A HPLC/ESI-MS METHOD DEVELOPED AND VALIDATED TO EVALUATE THE QUANTITY, IDENTITY, AND STABILITY OF THE ALKYLAMIDES IN ETHANOLIC EXTRACTS OF *SPILANTHES ACMELLA*

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

The proof of efficacy of herbal drugs has been a topic of controversy and debate in the medical community for several decades.[1] The value of double-blind, randomized, controlled studies to prove efficacy of herbal drugs has not completely gained full support by the medical community, although it has not yet been entirely abandoned.[1] In order to obtain reproducible results from such trials, it is necessary to establish a chemical standardization of the extract preparation.[2] To facilitate this standardization, there must be ways to qualitatively and quantitatively determine constituents and their concentrations in various extracts. It is also necessary to know which constituents are important in the biological activity of the extract. Thus, clinical trials and *in vitro* research concerning biological activity go hand in hand with chemical analysis; it is not possible to know which constituents are important without knowing which constituents are in the products being tested for efficacy.

Constituents and Therapeutic Use of *Spilanthes acmella*

A popular herbal preparation, *Spilanthes,* is used traditionally to treat mouth aliments, stammering, stomatistis, and throat complaints.[3] Several studies have demonstrated biological activity of *Spilanthes* species. Ratnasooriya and Pieris demonstrated that cold water extracts of the flowers of *Spilanthes acmella* possess antinociception activity

against persistent pain and antihyperalgesic activity.[4] Sukumaran and Kuttan observed chloroform extracts of *S. calva* inhibited tobacco induced mutagenesis on *Salmonella typhimurium*.[5]

Spilanthes' biological activity has primarily been attributed to constituents known as alkylamides, which this plant possesses in abundance. Alkylamides from *Spilanthes* have demonstrated strong diuretic[6] and insecticidal properties,[7] and alkylamides from other plants (specifically *Echinacea* species) have demonstrated *in vitro*[8, 9] and *in vivo*[10] immunomodulatory activity. Today, alkylamide containing extracts of *Spilanthes* are sold as dietary supplements by a number of herbal extract manufacturers, and the biological activity of these extracts and their constituents is an ongoing subject of research. Validated methods for analysis of alkylamides in complex *Spilanthes* extracts are needed to facilitate this research and to serve as the basis for quality control of commercial *Spilanthes* products.

Spilanthes is a native plant of the tropics of Africa and South America with yellow flower heads. *Spilanthes* is commonly referred to as the "toothache plant," a name that refers to the analgesic action of the alkylamide constituents. The species of interest in this research is *Spilanthes acmella* which is also known as *Acmella oleracea* and *Spilanthes oleracea*.

Alkylamide constituents are restricted to four families in the botanical kingdom.[11] Alkylamides with unsaturated acid residues can be classified as purely olefinic or acetylenic, which possess both olefinic and acetylenic linkages.[12] Purely olefinic alkylamides have been isolated from the four families, but the acetylenic alkylamides

have only been isolated from one family, *Asteraceae*.[12] Within the *Asteraceae* family, the purely acetylenic alkylamides are restricted to two tribes: *Anthemideae* and *Heliantheae*.[12] Both types of alkylamides have been reported from genera belonging to the *Heliantheae* tribe, such as *Heliopsis,*[13, 14] *Echinacea,*[8, 15-17] and *Spilanthes.*[3, 11, 13, 14, 18-25] The research presented here focuses on the chemical analysis of *Spilanthes* (family: *Asteraceae*, tribe: *Heliantheae*, subtribe: Ecliptinae.)[11]

Previously Published Methods for Analysis of Alkylamides from *Spilanthes*

Alkylamides have previously been detected in *Spilanthes*.[3, 11, 14, 20-26] However, there is relatively little research published on the identification of alkylamides in *S. acmella*, and no method exists for their quantification. Although there is currently no literature report of the quantitative analysis of alkylamides from *Spilanthes*, Nagashima and Nakatani used NMR spectroscopy and HPLC-MS with atmospheric pressure chemical ionization[11] and electron impact ionization[3] to determine structure of pungent alkylamides in extracts of *S. acmella*. They identified nine alkylamides, the most abundant of which was determined to be (2E,6Z,8E)-N-isobutyl-2,6,8-dectrienamide or spilanthol. Alkylamides have also been identified in several other species of *Spilanthes* including: *S. americana,*[25] *S. oppositifolia*,[26] *S. ocymifolia,*[20] *S.ciliata,*[22, 23] and *S. mauritiana.*[21] These studies demonstrate that an array of different alkylamides are present in *Spilanthes*, but none of them provide an approach for quantitatively evaluating alkylamide content of *Spilanthes* extracts.

Research Objectives

Our objectives in these studies were to evaluate the quantity, identity, and stability of the alkylamides in ethanolic extracts of *Spilanthes acmella*. To accomplish these objectives, we developed and validated a novel method for analysis of *Spilanthes'* alkylamides using HPLC coupled to electrospray ionization mass spectrometry (ESI-MS). This method demonstrates the power of HPLC/ESI-MS for both quantification and identification of plant constituents. In addition, the method described here will facilitate further assessment of the therapeutic potential of *Spilanthes* by providing an approach to monitor constituent concentration in extracts used for *in vitro* or *in vivo* studies. Finally, data indicating the stability of spilanthol in ethanolic extracts stored under various conditions is presented.

Overview of Techniques Used in this Research

The major analytical technique used in these studies is reversed-phase high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS) or reversed-phase high-performance liquid chromatography with UV photodiode array spectrophotometric detection (HPLC-PDA). HPLC is a chromatographic technique in which analyte resolution occurs when the components of the solution interact to different extents with the stationary phase.[27] In reversed phase HPLC, the stationary phase is non-polar and the mobile phase is polar. In this research, a column with a C18 alkyl group bonded to the silica surface is used, which constitutes a non-polar stationary phase. Separation of mixture components occurs based on their

relative attraction to the stationary (non-polar) phase with the most polar analytes eluting more rapidly than the less polar ones.[27]

We utilized preparatory scale HPLC-PDA to isolate the spilanthol standard. The use of the PDA detector allows the column effluent to be monitored for the analyte of interest based on its absorbance. For these experiments, the wavelength monitored was 254 nm, in the UV region. Alkylamides are able to absorb UV light due to the conjugated pi system of electrons along their unsaturated alkyl chains (see alkylamide structures in Figure 20). However, the use of the PDA detector provides very little structural information about analytes; therefore, additional methods of detection are needed for structural elucidation.

For identification and quantification experiments, the HPLC/ESI-MS is used. In this technique, the column effluent is directly interfaced to the ion trap mass spectrometer. Electrospray ionization used in this research accomplishes ionization from solution and is carried out at atmospheric pressure. The column effluent is passed through a metal capillary held at a high voltage in order to separate charged analyte molecules from their counter ions.[27] The solution protruding from the capillary becomes highly charged, and the coulombic repulsion of like charged ions eventually overcomes the surface tension of the solution and causes it to break up into a mist of highly charged droplets.[27] These droplets then undergo desolvation aided by drying gas (nitrogen) until the repulsive forces of the charges again exceed the cohesive forces of surface tension. At this point the droplet then fissions into a series of smaller droplets by a process known as a "coulombic explosion."[27] The droplets undergo these "coulombic explosions" until free

analyte ions are produced. These ions are then directed into the mass spectrometer through a series of focusing lenses. The ions are "held" in the ion trap, which consists of three hyperbolic electrodes. Two of these electrodes are endcaps and the third is a doughnut shaped ring electrode.[28] The "trap" contains helium gas at a pressure of typically, 1 mTorr, which reduces the kinetic energy of the ions trapped, thereby, contracting their trajectories toward the center of the trap.[27] These ions will remain in the trap until a RF voltage is applied to the ring electrode. This RF voltage makes the trajectory of ions with a particular m/z unstable and causes them to be ejected from the trap.[27] A complete mass spectrum can be obtained by varying the RF voltage in a systematic way so as to sequentially eject ions of increasing m/z from the trap. The mass spectra obtained with positive ion ESI-MS consist primarily of the protonated molecular ions (MH^+) of constituents of the solution. Salt clusters can also be detected, as can deprotonated species in the negative ion mode. ESI is a soft ionization technique, therefore, it causes little fragmentation and identification of the molecular ion is usually possible. This facilitates determination of the mass of the analyte of interest, which is a key element in the process of identification, but additional information is still necessary to obtain a positive identification of the analyte of interest.

Further structural elucidation and conformation of identity can be achieved by tandem mass spectrometry (MS-MS) analysis. A MS-MS spectrum is obtained by selecting and fragmenting the molecular ion of interest. The resulting scan gives a mass spectrum of the product ions produced from the decomposition of the molecular ion. In MS-MS analysis utilizing an ion trap mass spectrometer, the ion of interest is held in the trap

while all other ions are ejected. The isolated ion is then fragmented by collisionally activated dissociation due to interaction with excited helium gas in the trap. The resulting fragments are then ejected from the trap by varying the RF voltage, giving rise to a MS-MS (fragmentation) spectrum. For this research, data dependent MS-MS was used. Using data dependent MS-MS, the most abundant ion that elutes from the HPLC column at a given time can be trapped and fragmented, producing a MS-MS spectrum. If the precursor ions of interest are known, a list of ions of interest can be generated, so that the most abundant ion detected from the list at any given time will be subjected to MS-MS analysis. The MS-MS spectrum obtained can be utilized to confirm or determine the structure of the compound of interest. However, even though a great deal of information can be obtained from MS-MS spectra, the stereochemistry of the molecule can not be determined. In order to positively assign stereochemistry and compound structure, complementary techniques such as nuclear magnetic resonance (NMR) spectroscopy are essential.

NMR spectroscopy enables elucidation of the carbon backbone of an organic molecule and the environments of the hydrogens attached.[29] For this research both ^{1}H-NMR and ^{13}C-NMR were utilized. NMR spectrometers measure the amount of energy absorbed by a nucleus of an atom when it is excited from its lowest energy spin state to the next higher one.[29] For ^{1}H-NMR, the amount of energy absorbed by the proton's nucleus is influenced by neighboring electrons, the more electrons the more shielded the proton and hence, the more energy required to flip its spin. This variation in energy absorbed gives rise to chemical shifts which can be different for various protons in a

molecule and are characteristic of particular structural features.[29] As with electrons influencing the energy absorbed by protons, the magnetic field of different types of protons can also influence each other resulting in spin-spin splitting. By calculating the separation between adjacent lines in a signal, referred to as the coupling constant, the position (i.e. cis or trans) of the hydrogens to one another can be determine. ^{13}C-NMR works essentially the same way except there is no spin-spin splitting observed. ^{13}C-NMR enables the determination of the magnetic environment of the carbon atoms in the molecule. These carbon atoms are also prone to shielding by neighboring electrons; however, these effects are much greater for carbon than for the protons because they are directly connected to the neighboring carbon. By evaluating the chemical shifts of the carbon atoms, their connectivity can be discerned and, hence, the backbone of the molecule can be determined.

Method Validation

The HPLC/ESI-MS method developed and used to quantify alkylamides in ethanolic extracts of *S. acmella* was validated according to guidelines given by the International Committee of Harmonisation (ICH) section Q2 (R1.)[30] The aspects investigated were linearity, range, precision [a) repeatability, b) intermediate precision], and limits of detection and quantification.

Linearity of an analytical procedure is the concentration range in which the analyte response is directly proportional to the concentration of the analyte in the sample. The working range of the method is determined by confirming that the method provides an acceptable degree of linearity and precision for a range of standard concentrations.[30]

Both the linearity and range are determined by evaluating a range of concentrations of a standard of the analyte of interest.

The closeness of agreement (degree of scatter) between a series of measurements obtained from sampling the same sample under the same conditions is expressed as the precision of an analytical method.[30] The method precision was considered at two levels: repeatability and intermediate precision. Precision is expressed as the standard deviation of a series of measurements. Repeatability evaluates precision of samplings under the same conditions over a short interval of time. The effects of random events on the analytical procedure are evaluated with intermediate precision. Intermediate precision is determined similarly to the repeatability, but the analysis is conducted on data collected over multiple days. The standard deviation and relative standard deviation are then determined from collected data.

The least amount of analyte in a sample which can be detected but not necessarily quantified as an exact value is the limit of detection (LOD) of an analytical procedure.[30] The detection limit can be determined by several approaches such as: visual inspection, signal-to-noise, standard deviation of the response and the slope, and calibration curve. The smallest amount of analyte in a sample that can be reliably and accurately quantified is the limit of quantitation (LOQ) for an analytical procedure.[30] The same approaches used for LOD determination (visual inspection, signal-to-noise, standard deviation of the response and the slope, and calibration curve) can be used to determine LOQ.

Antibacterial Assay

A recent study investigating the antifungal potential of flower head extract of *S. acmella* showed that the extract possesses fungi toxic activity against potential human pathogens *Asperigillus niger* and *A. paraciticus*, and agricultural pathogens *Fusarium oxysporium* and *F. moniliformis*.[31] In another study, chloroform, methanol, and aqueous extracts of various plants including *Spilanthes* were tested for antibacterial activity against important pathogenic bacteria including *Staphylococcus aureus*. Only the chloroform extract showed any antibacterial activity, and that extract was only toxic to *Streptococcus mutans*.[32] In an ethnopharmacological survey, extracts of six East African medicinal plants including *Spilanthes mauritiana* were tested against 105 strains of bacteria. The minimum inhibitory concentration and minimum bactericidal concentrations were determined to be $\geq$ 8mg/mL for the methanolic extracts of *S. mauritiana*, which indicates low antibacterial activity.[33] However, the compounds responsible for the antifungal or antibacterial activity seen were not specified. One of our goals in the studies described here is to evaluate antibacterial activity of isolated alkylamides from *S. acmella*, including spilanthol, as well as complex *S. acmella* extracts. This should show if any antibacterial activity observed is due solely to the alkylamides or if it is due to a combined effect with other constituents in the extracts. The complex extract and isolated spilanthol will also be tested in combination with berberine to see if constituents of *S. acmella* have multidrug resistance pump inhibitory activity.[34]

CHAPTER II

EXPERIMENTAL

Preparation and Storage of Extracts

Fresh whole plants of *S. acmella* were extracted using a range of ethanol concentrations. The extraction was accomplished by blending the plants in the appropriate solvent (0, 10, 25, 50, 75, or 95% ethanol) at a ratio of 2 mL solvent: 1g plant material. The extracts were then allowed to macerate for 14 days at ~4°C and the solvent was removed using a hydraulic press. These extracts were stored at -20°C. From the 95% ethanol extract, the spilanthol standard used to validate the HPLC/ESI-MS method was isolated.

In order to study the stability of spilanthol in a *S. acmella* extract, fresh whole plants of *S. acmella* were obtained from Horizon Herbs (Williams, OR) and a voucher specimen was pressed and stored. The fresh plant was washed thoroughly and weighed to the nearest 1 g. A total of 577 g plant material was used. The solvent used for the extraction consisted of 75% ethanol and 25% water. This extraction solvent was prepared by making a mixture consisting of 21% water (purified with a Barnstead Nanopure Diamond, D11931, Barnstead International, Dubuque, IA) and 79% ethyl alcohol USP (190 proof, 95 % ethanol, AAPER Alcohol and Chemical Co., Shelbyville, KT.) A 2:1 extract (2 mL solvent: 1 g plant material) was prepared with a final ethanol

content of 60% (accounting for water from the plant material as well as the solvent.) The plant material and solvent were thoroughly blended using a commercial blender (Waring Commercial 51BL30, Torrington, CT) and allowed to macerate for a period of 7 days. The extract was then expressed from the plant using a hydraulic press and filtered under vacuum with a 0.2 μm nylon filter to remove any particulates. Three aliquots of the extract (10 mL in 15-mL polypropylene tubes, Fisher Scientific, Pittsburgh, PA) were stored at each of the following conditions -80°C, -20°C, and room temperature. The remaining extract was stored at -20°C.

Solid Phase Extraction

The lipophilic alkylamides in the 95% ethanol extract were separated from the hydrophilic plant matrix by SPE using an eluent ethanol content from 45 to 100%. Twelve fractions were collected overall. These fractions were analyzed by HPLC/ESI-MS to confirm presence of and tentatively identify possible alkylamides.

Analysis of Extracts with HPLC/ESI-MS

Analyses of spilanthol content were carried out with reversed phase HPLC coupled to ESI-MS. An HP1100 (Agilent, Palo Alto, CA) HPLC system was used for the separation. The chromatographic column used was a short, narrow bore C18 column (50 mm x 2.1 mm, 3 μm particle size, 110 Å pore size, Prevail packing, Alltech, Deerfield, IL) equipped with a 0.5 μm precolumn filter (MacMod Analytical, Chadds Ford, PA). An isocratic separation was conducted with mobile phase composition of 50% A and 50% B (where A = 1% acetic acid in nanopure water and B = HPLC grade acetonitrile).

A flow rate of 0.2 mL/min was used, the injection volume was 10 µL, and the total analysis time was 22 min.

The outlet of the HPLC column was connected directly to an ion trap mass spectrometer (LCQ Advantage, Thermo Finnigan, San Jose, CA). Mass spectrometric detection was carried out in the positive ion mode with a scan range of 100 -1000 m/z. The mass spectrometer was operated with the following parameters: a capillary temperature of 275°C, a sheath gas pressure of 20 arb, and spray, capillary, and tube lens voltages of 4.5 kV, 3 V, and 60 V, respectively.

Isolation of Spilanthol

The standard used to quantify and validate the HPLC/ESI-MS method was isolated from a 95% ethanol *S. acmella* extract using preparatory scale HPLC-PDA with fraction collection. SPE was used to remove the hydrophilic compounds from a 50 mL aliquot (diluted to 100 mL with deionized water) of the 95% ethanolic extract of *S. acmella*. The lipophilic alkylamides eluted with 100 mL of a 95% ethanol solution. The solvent from 12 mL of the SPE fraction was removed under vacuum and reconstituted in 2 mL of 95% ethanol. The reconstituted sample was used for HPLC-PDA fraction collection to obtain isolated spilanthol. An HPLC HP1100 (Agilent, Palo Alto, CA) with a UV/VIS photodiode array detector and a Haisil 100 C18 column (150 mm x 100 mm, 5µm particle size, 100 Å pore size, Higgins Analytical, Mountain View, CA) was used. The injection volume was 500 µL and the flow rate was 2.0 mL/min. The separation was accomplished isocratically using the same solvent composition described above. Absorbance was monitored at 254 nm, and a peak that eluted at 16.0 min was collected.

The collected fractions from five separations were combined in a pre-weighed centrifuge tube and the solvent was removed under vacuum to determine dry weight. The sample was then re-dissolved in ethanol at a concentration of 1.0 mg/mL (4.5×10^{-3} M) to make the stock standard solution of spilanthol.

Purity of Spilanthol Standard

The purity of the solution was estimated by HPLC/ESI-MS and HPLC-PDA by calculating the % peak area in the chromatogram corresponding to spilanthol. The purity of the spilanthol peak obtained using HPLC-PDA was also evaluated by observing the absorbance spectra obtained at multiple points across the peak. If the peak is due to one compound then the absorbance spectra should be identical across the peak.

Quantification and Stability of Spilanthol in Ethanolic Extracts

The stock solution of spilanthol (isolated from *S. acmella* as described above) was serially diluted to make five standards at concentrations of 1.4 μM, 4.5 μM, 14 μM, 45 μM, and 140 μM. The calibration standards were analyzed using HPLC/ESI-MS as described earlier. A calibration was generated by plotting the log of the sum of the peak areas of the selected ion chromatograms for the spilanthol ions versus the log of the concentration. Because spilanthol forms a dimer in the gas phase, the peak area for m/z 443 (proton bound dimer) was doubled and added to the peak area of m/z 222 (protonated molecular ion) to account for all of the spilanthol response.

The replicate extract samples stored under various conditions were also analyzed by HPLC/ESI-MS. Prior to analysis, an aliquot (4 μL) of each extract was diluted to 1.5 mL in 95% ethanol to provide a sample within the linear range of the calibration curve. The

samples were analyzed at various time points to monitor degradation of spilanthol. For each analysis, calibration standards were analyzed in the same run with the samples to minimize errors due to day-to-day variability in instrument response.

Identification of Alkylamides

Tentative identification of eight alkylamides was achieved by comparison of molecular ions and MS-MS fragmentation patterns. The molecular ion values were compared to literature values.[3, 11] The MS-MS fragmentation patterns obtained with HPLC/ESI-MS and activated dissociation in the ion trap with a collision energy of 35% were compared to expected fragmentation of alkylamides.[35]

The MS-MS spectra for the alkylamides were obtained by utilizing a data dependent scan (Xcalibur ver. 1.4 SR1) and collisionally induced dissociation (CID) within the ion trap of the mass spectrometer. A list of precursor ions was chosen for the data dependent scan based on published masses of alkylamides and consisted of the following m/z values: 204, 232, 222, 246, 258, 236, 260, 230, 248, 252, and 268. Two analyses of the *S. acmella* extract were conducted using this list of ions, one in which the most intense ion from the list was fragmented at any given time, and one in which the MS-MS scan was collected for the second most intense ion from the list. This approach facilitated collection of MS-MS spectra for both the major and minor alkylamides present in the extract.

Positive identification of spilanthol was confirmed based on the mass of the molecular ion, its MS-MS fragmentation pattern, its absorption maxima (λ_{max}) and spectrum, and comparison of ^{1}H-NMR and ^{13}C-NMR shifts to literature values.[11, 35] For

NMR analysis, a 3.4 mg of purified spilanthol was dissolved in 700µL of $CDCl_3$ and analyzed on a Jeol ECA 500MHz NMR spectrometer (Peabody, MA, USA.) A 10 mg/mL spilanthol standard was analyzed by HPLC-PDA as described earlier to obtain an absorption maxima and spectrum.

Method Validation

Linearity was determined initially by visual inspection of a calibration curve covering a large concentration range. Once a preliminary linear range was determined, five concentrations encompassing the range were analyzed in triplicate. A regression line was calculated by method of least squares on the linear portion of the calibration curve. The correlation coefficient, y-intercept and slope of regression line were then evaluated.

The external standard calibration method was employed on five standard solutions analyzed in triplicate. A calibration curve was constructed by plotting the log of the sum of corresponding peak areas of the selected ion chromatograms versus log of analyte concentration. The mean peak area from triplicate analyses of each sample was used to construct this curve. A regression line was calculated by method of least squares on the linear portion of the calibration curve to determine linearity.

The *S. acmella* standards were used to determine linear dynamic range, repeatability, and intermediate precision. The linear dynamic range was determined by analyzing standards ranging from 0.23 µM to 1400 µM in triplicate and plotting a calibration curve. The concentrations that gave a linear regression line and acceptable precision were used as a measure of the range. Repeatability was evaluated by analyzing the five spilanthol standards in triplicate and determining the standard deviation and

relative standard deviation of the back calculated concentration for those triplicate analyses. Intermediate precision was evaluated by analyzing the five standards in triplicate over a period of three days. The standard deviation and relative standard deviation for the intermediate precision were calculated using the average back calculated concentration for each of the three days for each standard concentration.

The approach that was utilized for determination of LOD and LOQ for these analyses was based on signal-to-noise because the HPLC/ESI-MS exhibits baseline noise. The signal-to-noise ratio was determined by comparing measured signals from samples with low analyte concentrations with those of a blank sample. The LOD was determined as the concentration that would give a signal-to-noise ratio of 3:1 and the LOQ was the concentration calculated to give signal-to-noise ratio of 10:1.

Antibacterial Assay

The antibacterial activity was tested by the agar diffusion method with top agar. Sterile filter paper disks (Whatman no. 41; 9 cm in diameter) were soaked with 10µL of sample and allowed to air dry. The complex *S. acmella* extract and isolated spilanthol were tested. The complex extract was a 75% ethanolic extract of fresh whole plants of *S. acmella* and was also concentrated 10 fold and analyzed. The concentrations of the isolated spilanthol tested were 1, 5, or 10 mg spilanthol per mL of ethanol. All samples were also tested in combination with 5µg/mL berberine (Sigma) to see if they had multidrug resistance pump inhibitory activity.[34] Disks were applied onto the surface of 1.5% Bacto ™ agar plates topped with a layer of 0.75% Bacto ™ agar seeded with a 24 hour culture of *Staphylococcus aureus* in Muller Hinton Broth (Fisher). The plates were

incubated at 37°C for 24 hours. A 30µg kanamycin (Fisher) antibiotic disk was used as a positive control. The antibacterial activity was evaluated by measuring the inhibition zone formed around the disk.

CHAPTER III

RESULTS AND DISCUSSION

Solid Phase Extraction

The results of SPE showed that the alkylamides eluted in fractions 2 through 11 with an eluent ethanol content of 45% to 60%. None of the fractions showed complete separation of the molecular ion with an m/z 222, spilanthol, from the other possible alkylamides present, and further purification with HPLC-PDA with fraction collection was employed. Figures 1-10 show the base peak chromatograms obtained using HPLC/ESI-MS for the SPE fractions 2-11 of *S. acmella*. For all the SPE figures, the top chromatogram represents the entire time period, while the lower chromatogram is an enlargement of the possible alkylamides. Table 1 summarizes the molecular ion m/z of the alkylamide and the fraction in which it elutes.

Table 1: Summary of SPE fraction contents.

SPE Fraction	MH^+ (m/z)
2	204, 268, 230, 232
3	268, 230, 232, 222 (spilanthol)
4	230, 232, 443 (proton bound dimer of spilanthol)
5	443, 236
6	222, 236
7	222, 236, 248
8	222, 236
9	236
10	236
11	236, 248

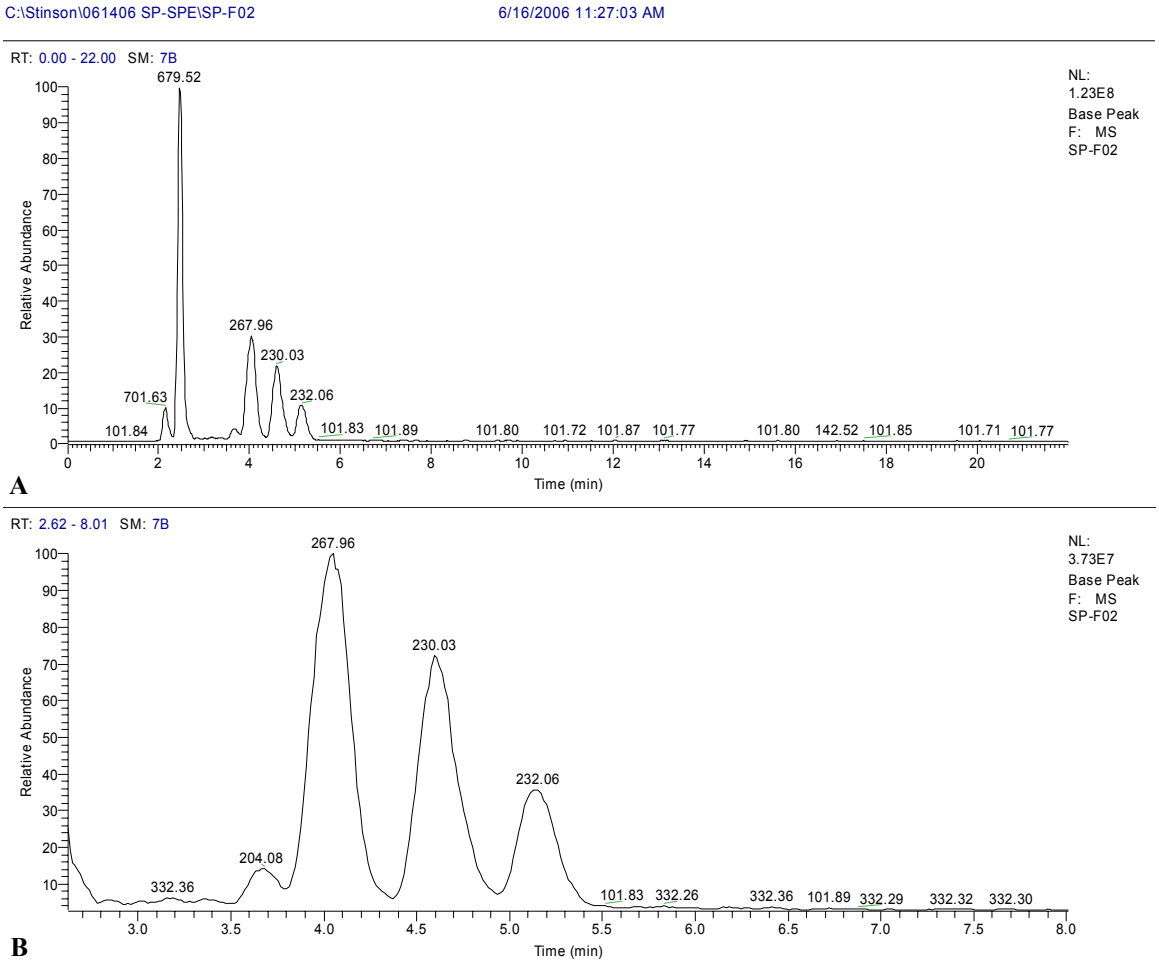


Figure 1: a) Base peak chromatogram of SPE fraction 2 of *S. acmella* obtained using HPLC/ESI-MS, b) base peak chromatogram zoomed in on peaks of interest from time 2.5-8 min.

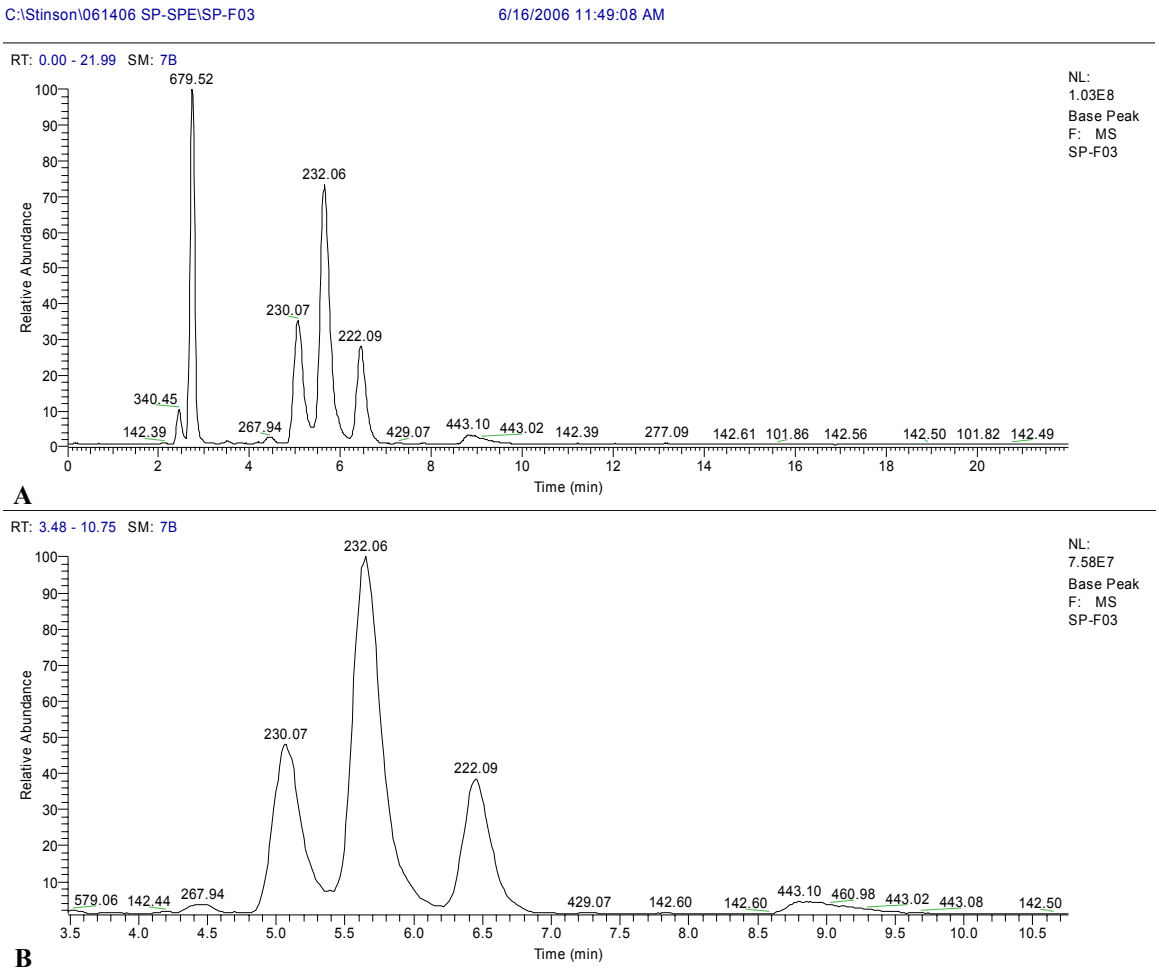


Figure 2: a) Base peak chromatogram of SPE fraction 3 of *S. acmella* obtained using HPLC/ESI-MS, b) base peak chromatogram zoomed in on peaks of interest from time 3.5-11 min.

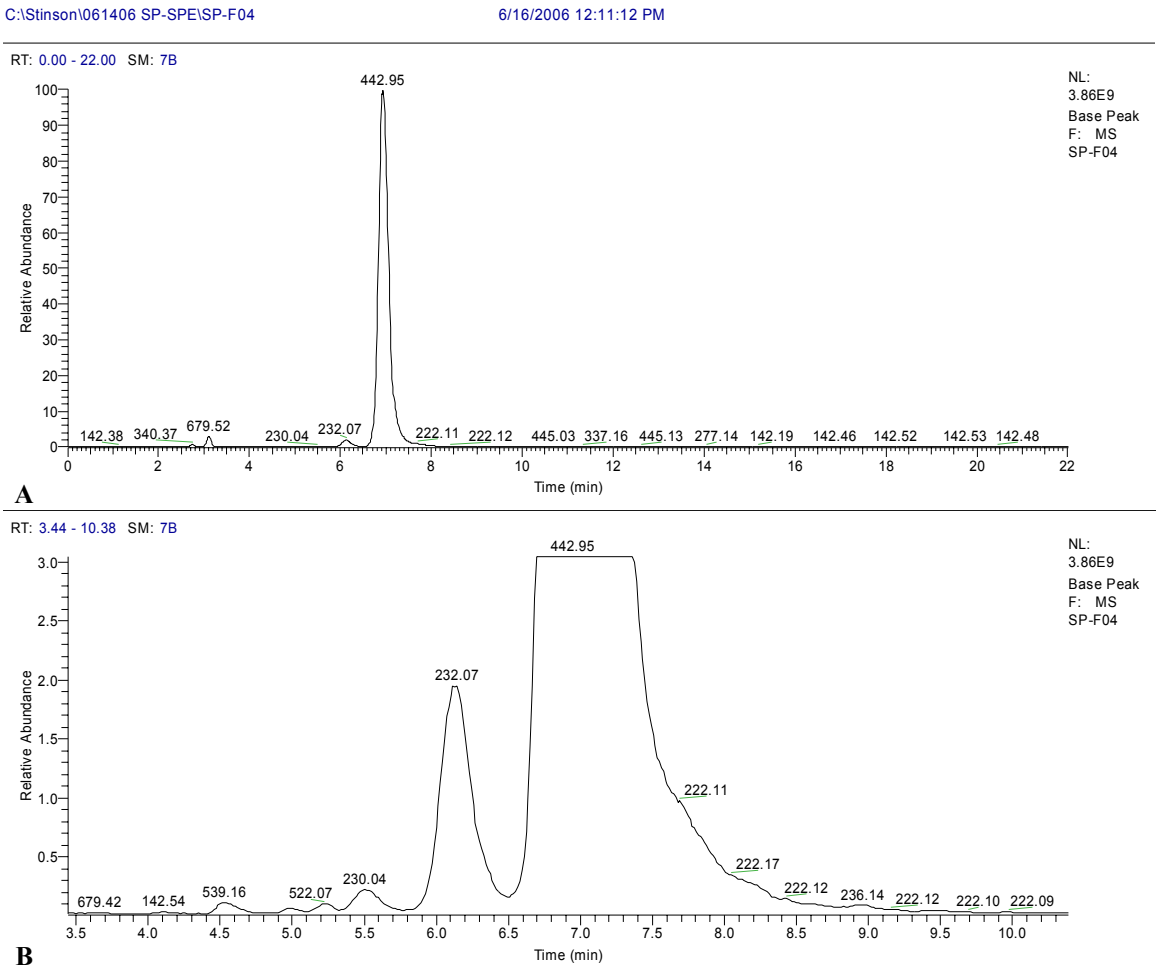


Figure 3: a) Base peak chromatogram of SPE fraction 4 of *S. acmella* obtained using HPLC/ESI-MS, b) base peak chromatogram zoomed in on peaks of interest from time 3.5-10 min.

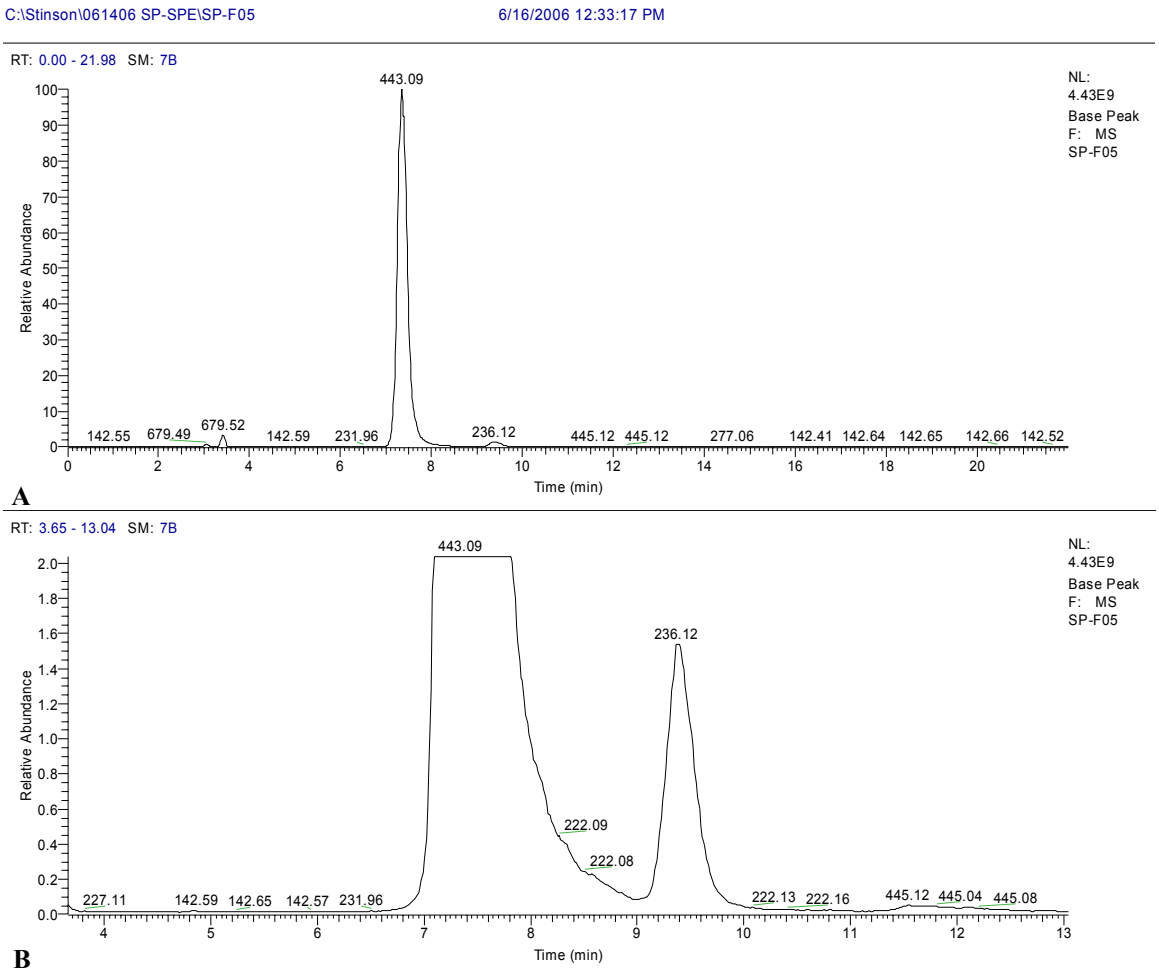


Figure 4: a) Base peak chromatogram of SPE fraction 5 of *S. acmella* obtained using HPLC/ESI-MS, b) base peak chromatogram zoomed in on peaks of interest from time 4-13 min.

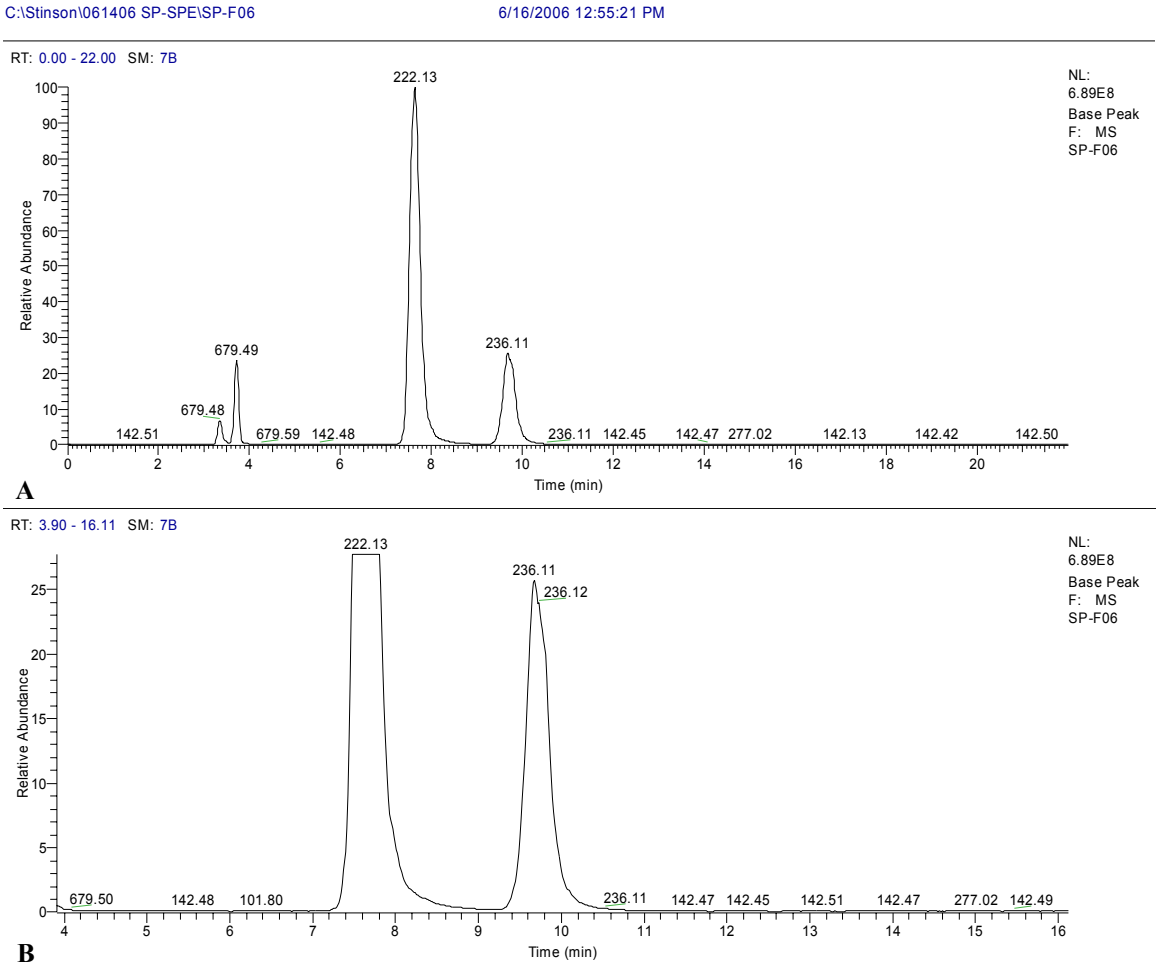


Figure 5: a) Base peak chromatogram of SPE fraction 6 of *S. acmella* obtained using HPLC/ESI-MS, b) base peak chromatogram zoomed in on peaks of interest from time 4-16 min.

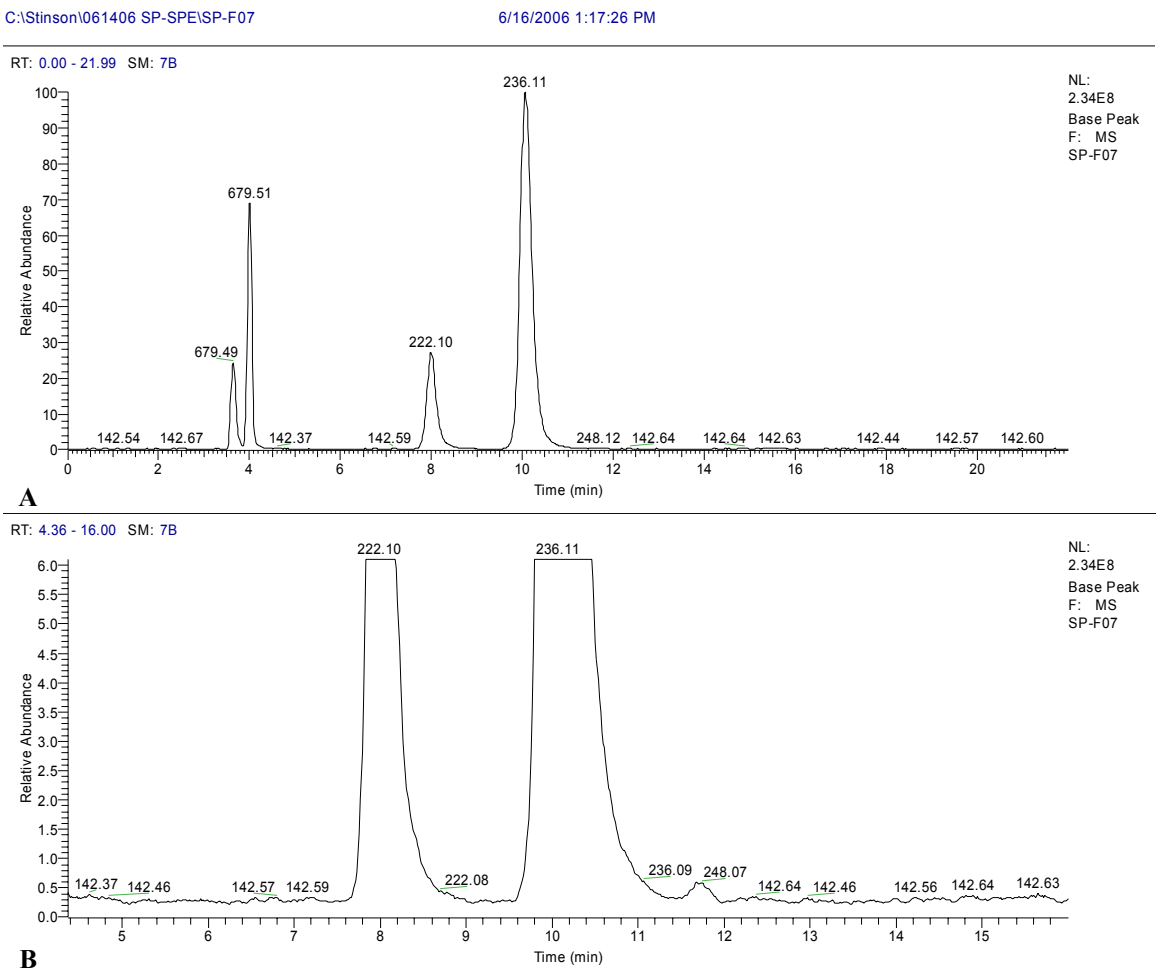


Figure 6: a) Base peak chromatogram of SPE fraction 7 of *S. acmella* obtained using HPLC/ESI-MS, b) base peak chromatogram zoomed in on peaks of interest from time 5-15 min.

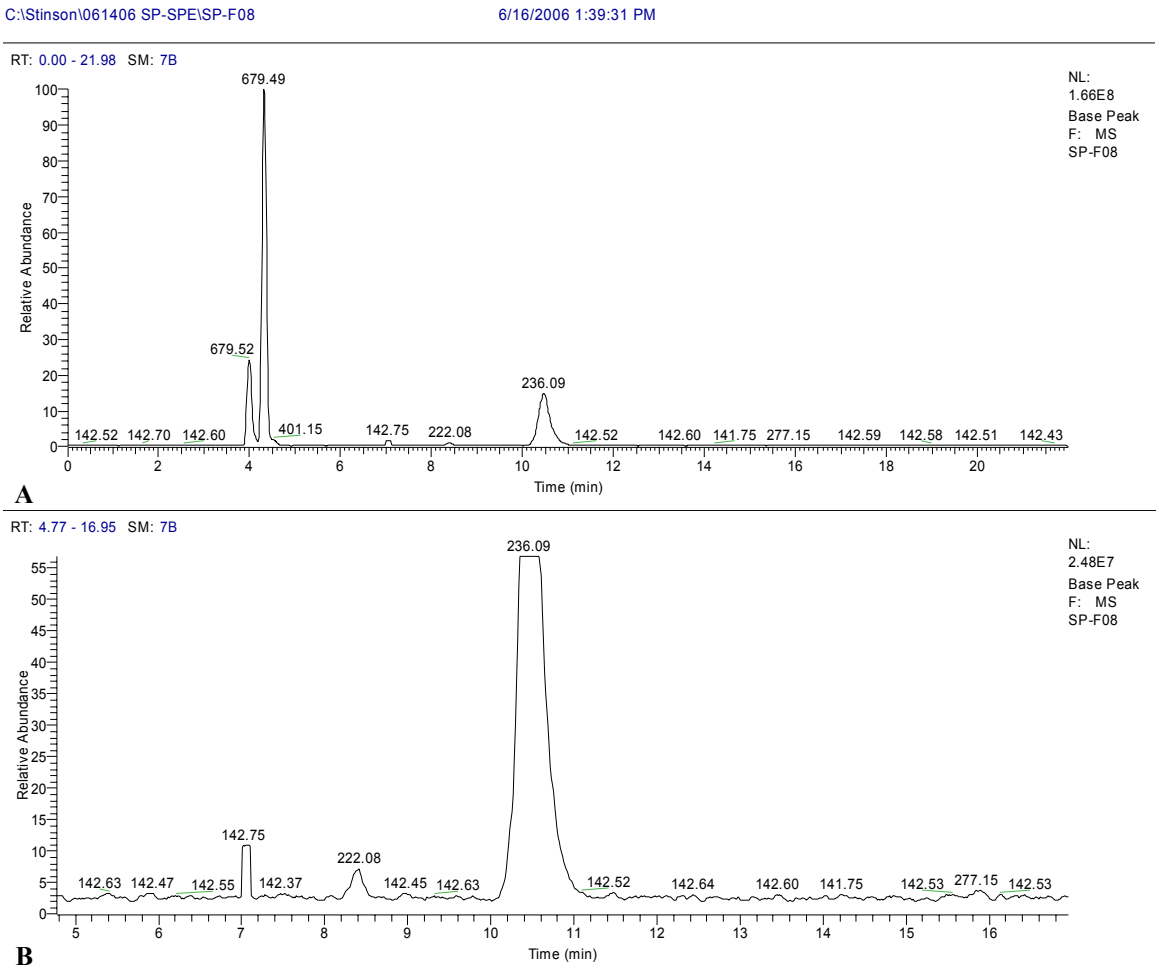


Figure 7: a) Base peak chromatogram of SPE fraction 8 of *S. acmella* obtained using HPLC/ESI-MS, b) base peak chromatogram zoomed in on peaks of interest from time 5-16 min.

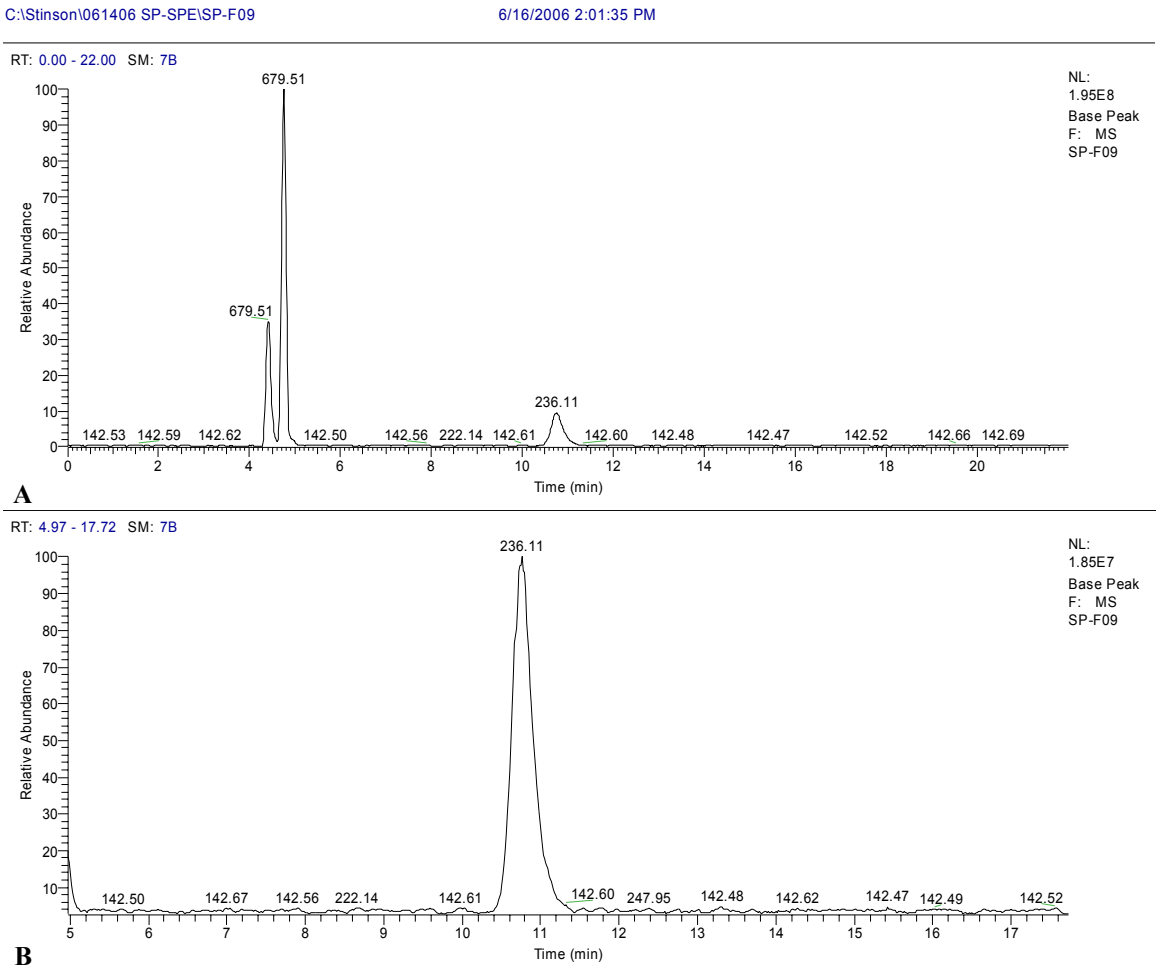


Figure 8: a) Base peak chromatogram of SPE fraction 9 of *S. acmella* obtained using HPLC/ESI-MS, b) base peak chromatogram zoomed in on peaks of interest from time 5-17 min.

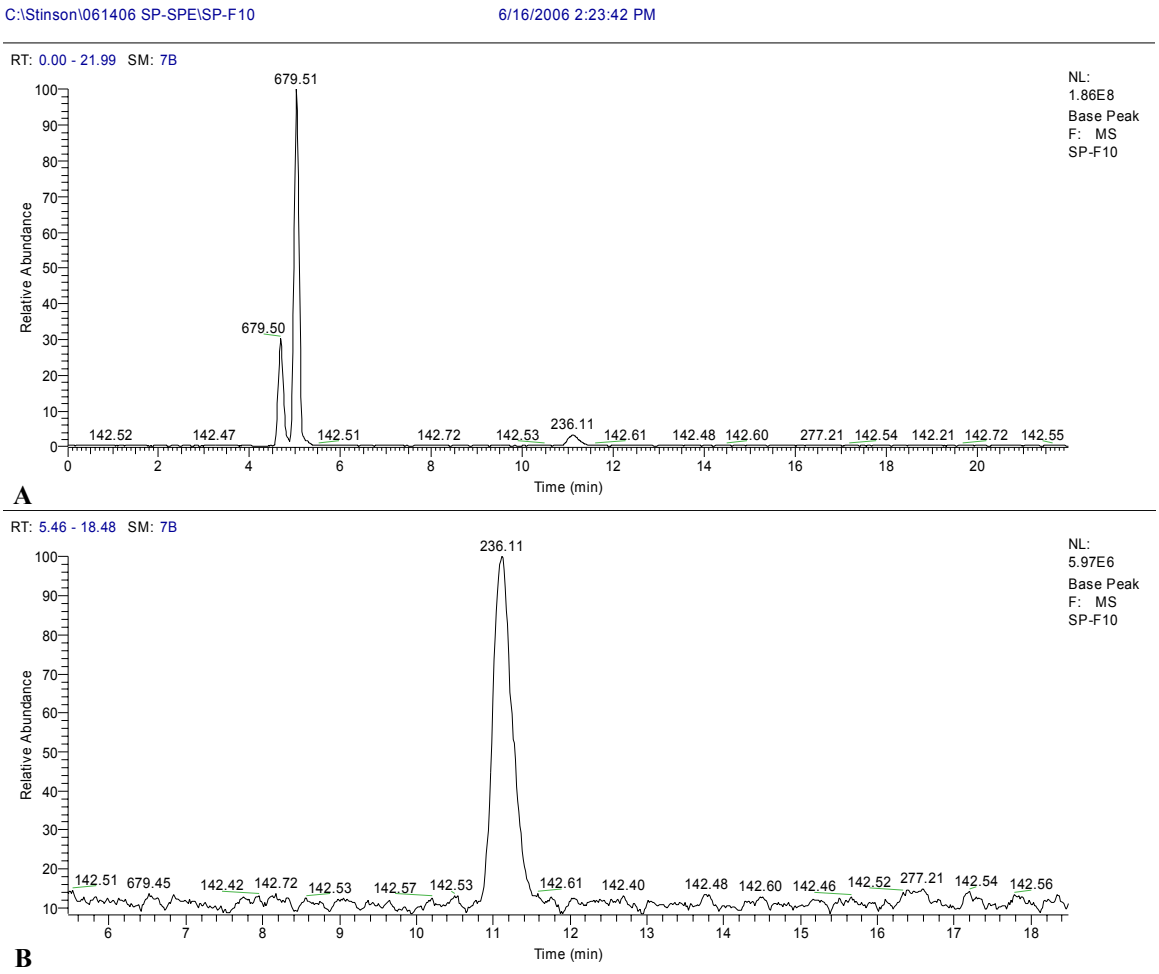


Figure 9: a) Base peak chromatogram of SPE fraction 10 of *S. acmella* obtained using HPLC/ESI-MS, b) base peak chromatogram zoomed in on peaks of interest from time 6-18 min.

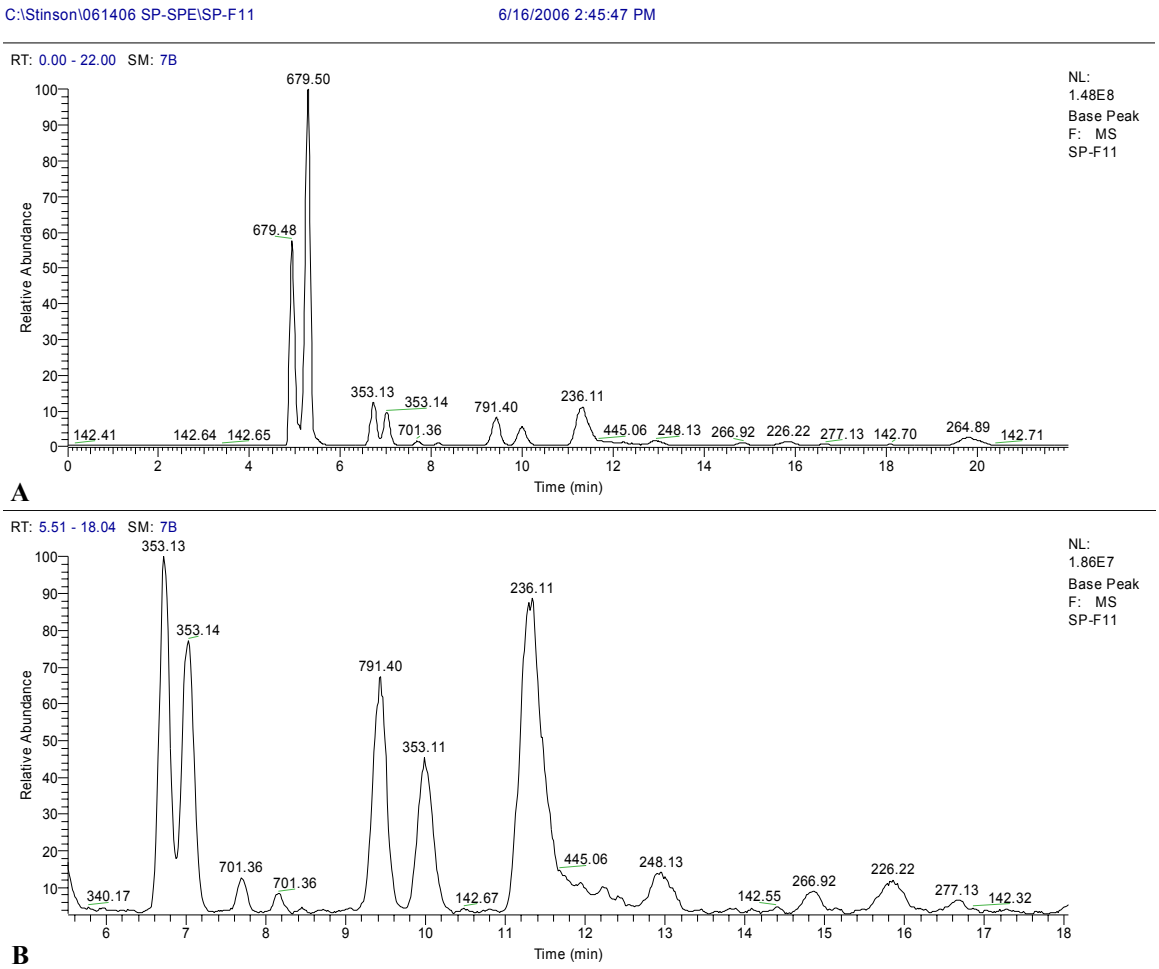


Figure 10: a) Base peak chromatogram of SPE fraction 11 of *S. acmella* obtained using HPLC/ESI-MS, b) base peak chromatogram zoomed in on peaks of interest from time 6-18 min.

Isolation of Spilanthol

The SPE method did not afford isolated spilanthol, so HPLC-PDA with fraction collection was employed to obtain a more highly purified sample. Figure 11 shows a representative chromatogram of the SPE fraction used for peak collection. The peak that

eluted at 16.0 minutes was collected and the solvent removed under vacuum to afford isolated spilanthol.

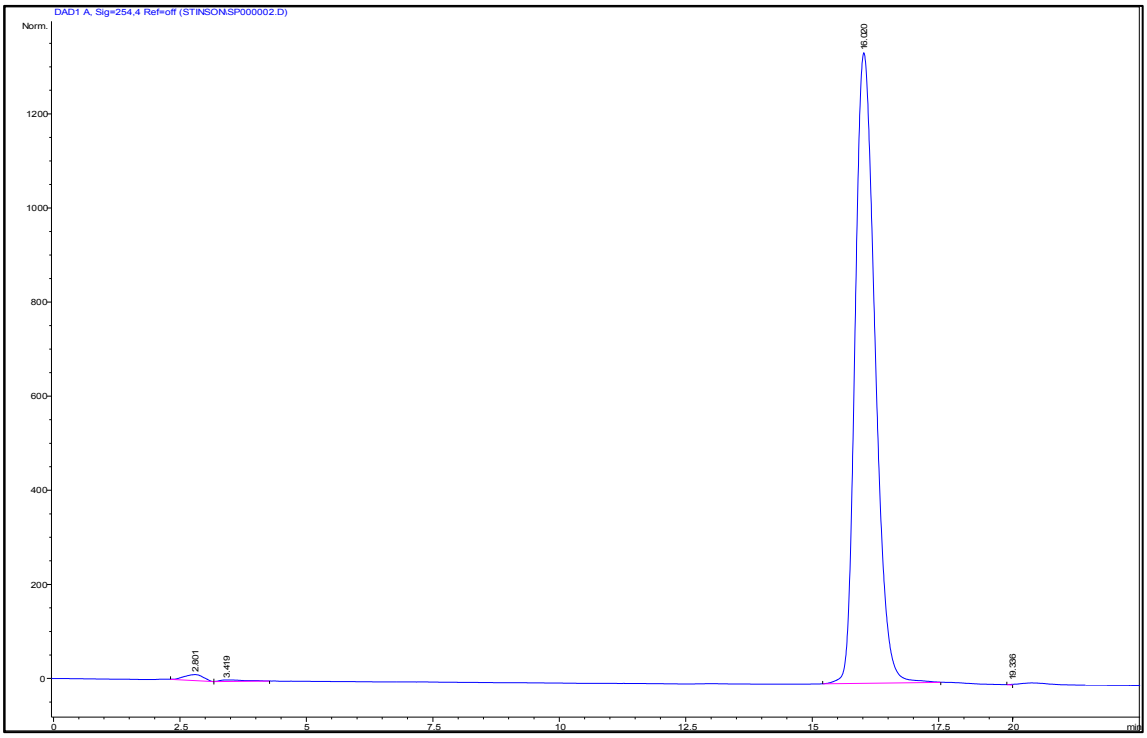


Figure 11: HPLC chromatogram of SPE fraction used for peak collection to isolated spilanthol generated using HPLC with photodiode array spectrophotometric detection.

Purity of Spilanthol Standard

The purity of the isolated spilanthol solution was estimated by HPLC-PDA and HPLC/ESI-MS. Figure 12 shows a blank (ethanol) chromatogram and a spilanthol standard chromatogram obtained using HPLC-PDA. There are no co-eluting peaks with the peak of interest, but there appears to be a slight impurity at the tail end of the

spilanthol peak. The purity of the spilanthol solution was estimated to be 97% using HPLC-PDA.

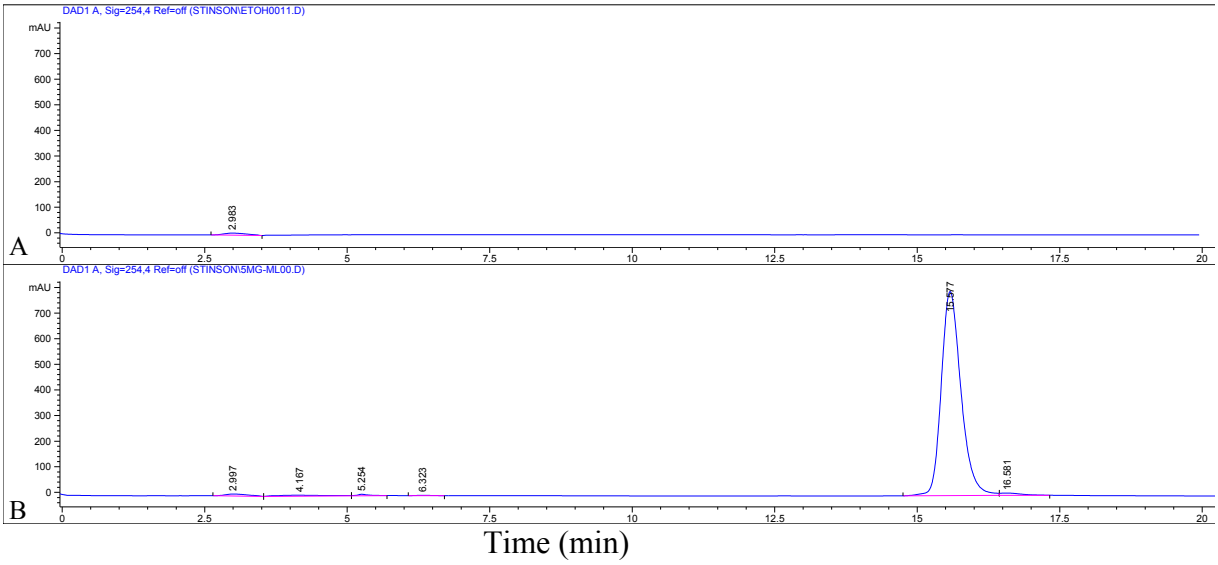


Figure 12: HPLC chromatograms of a) ethanol blank and b) spilanthol standard generated using HPLC with photodiode array spectrophotometric detection.

Figure 13 shows the base peak chromatograms for an ethanol blank (top) and a spilanthol standard (bottom) obtained using HPLC/ESI-MS. There appears to be no co-eluting peak with spilanthol, however, there are a few peaks corresponding to impurities. The purity of the spilanthol solution was estimated to be 88% using HPLC/ESI-MS.

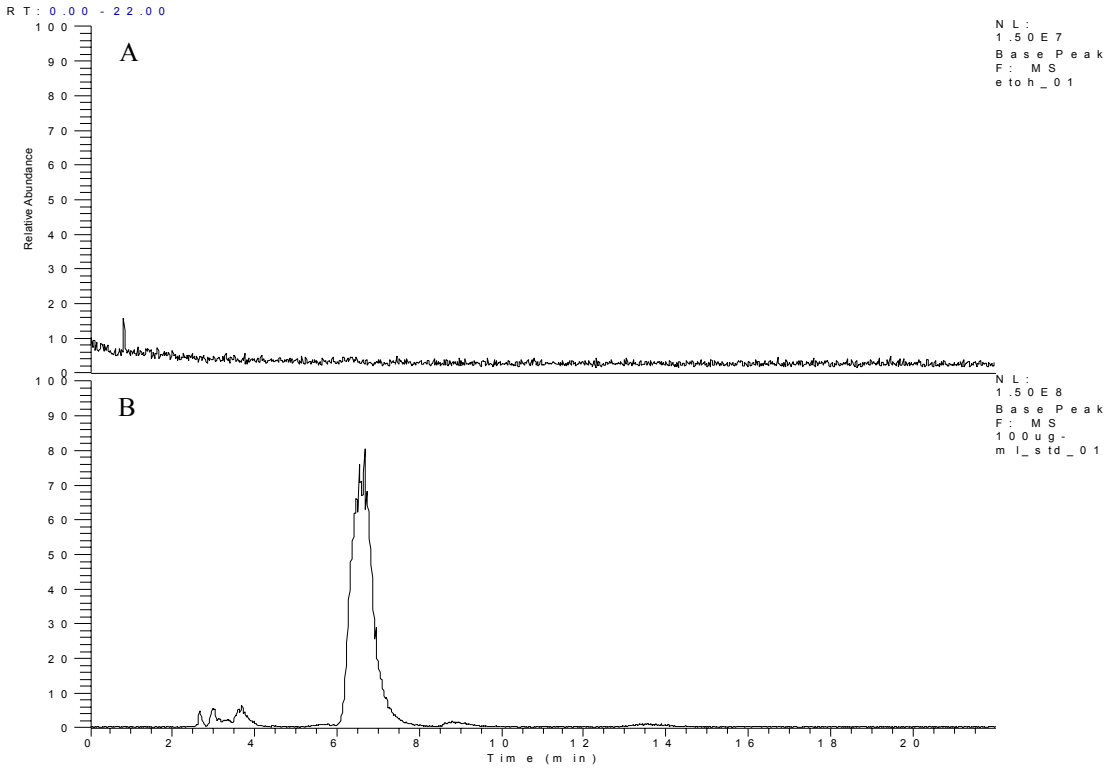
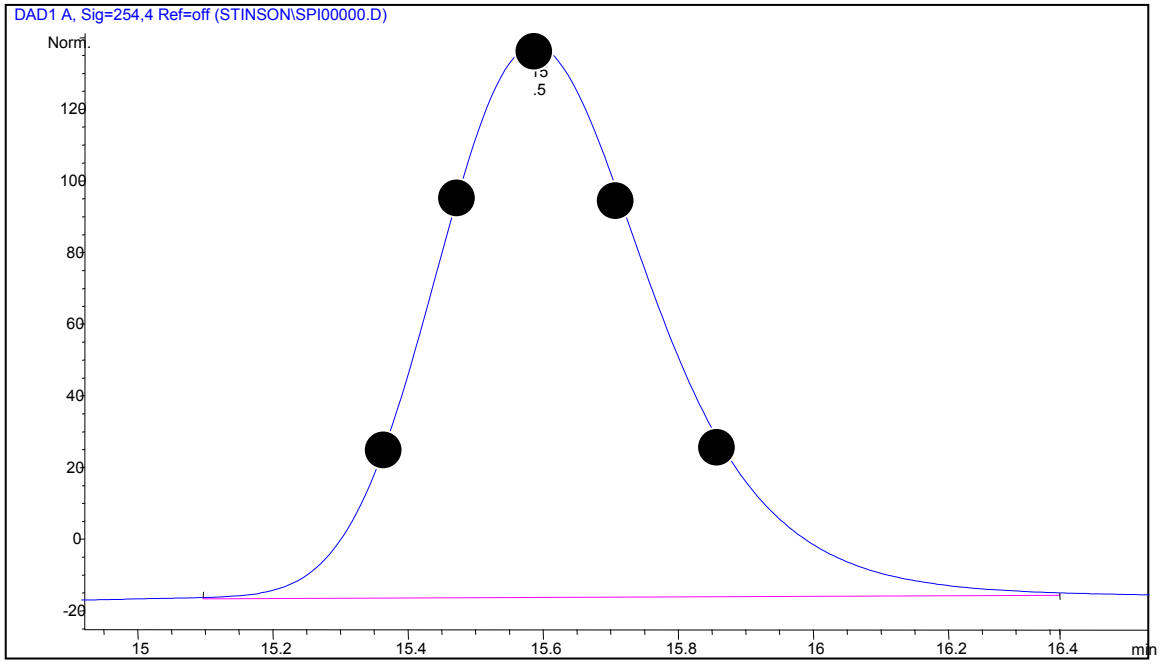
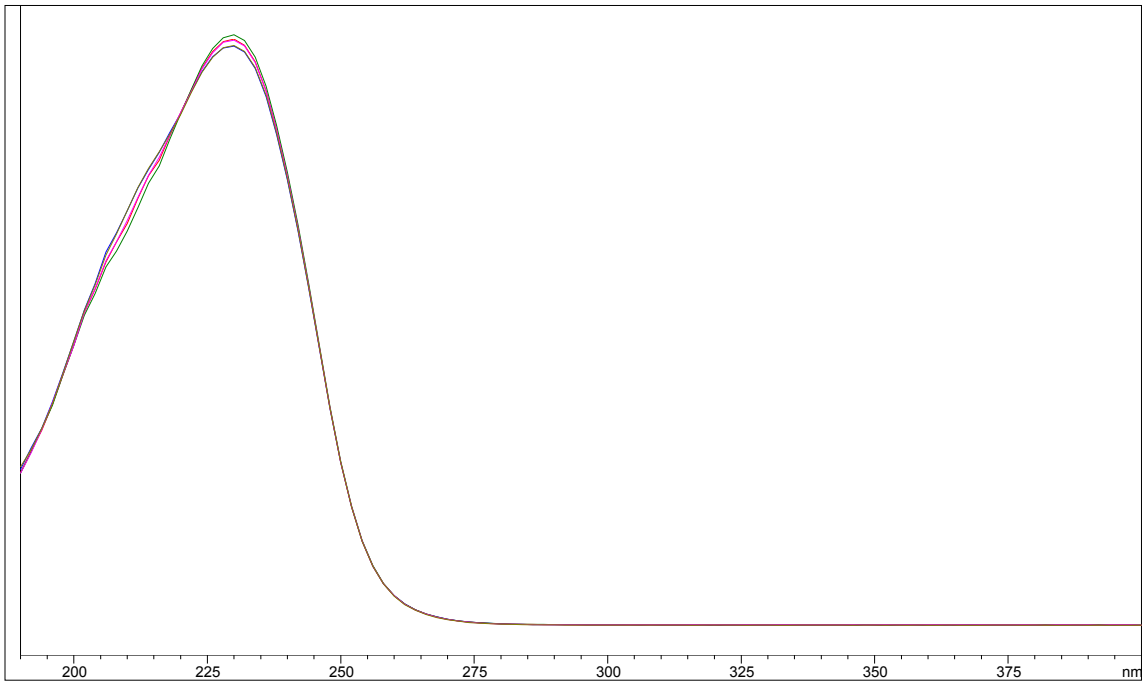


Figure 13: Base peak chromatogram of a) ethanol blank and b) spilanthol standard generated using HPLC/ESI-MS.

The purity of the spilanthol peak obtained using HPLC-PDA was also evaluated by acquiring the absorbance spectra at multiple points across the peak. Figure 14a shows where the absorbance spectra were taken on the chromatogram and 14b shows the overlay of those absorbance spectra. The similarity of the spectra shows that the peak is primarily due to spilanthol. However, there could be compounds that do not absorb light that co-elute or there could be compounds that are in such small quantities their absorbance spectra can not be distinguished from the spilanthol absorbance spectra.



A) **Time (min)**



B) **Wavelength (nm)**

Figure 14: a) Chromatogram of spilanthol showing points where UV-VIS spectra were acquired. b) UV/VIS absorbance spectra overlaid for spilanthol peak.

Quantification and Stability of Spilanthol in Ethanolic Extracts

The HPLC/ESI-MS method was employed to compare concentrations of alkylamides in extracts prepared from fresh whole plants of *S. acmella* using a range of ethanol concentrations for extraction. The spilanthol content was observed to increase with increasing ethanol concentration in the extraction solvent, and the most concentrated extract had a spilanthol concentration of $(7 \pm 1) \times 10^2$ μg/mL. Figure 15 shows the spilanthol content of the extracts analyzed.

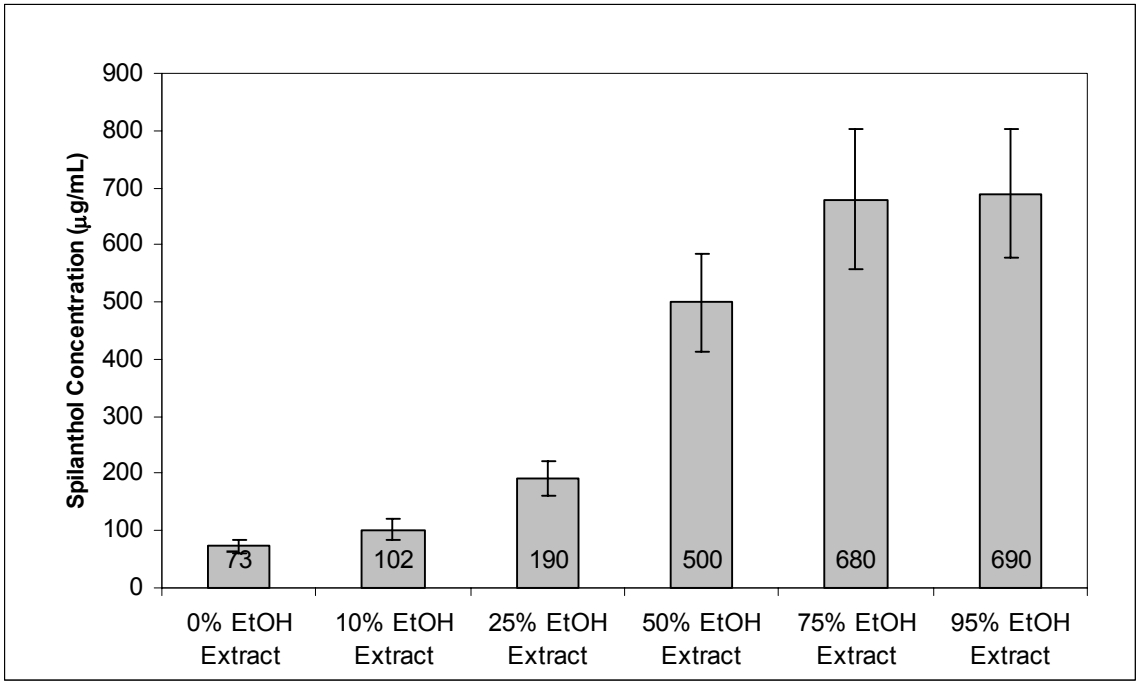


Figure 15: Spilanthol concentration in ethanolic extracts of *S. acmella*. Error bars represent +/- standard deviation which was calculated using linear regression.

The HPLC/ESI method was also employed to evaluate the stability of spilanthol in a 75% ethanolic extract of *S. acmella* over a period of six and a half months. Results of the stability study are shown in Figure 16. Data points represent the average of one injection of three replicate samples for each storage condition. The diamonds, squares, and triangles represent the three storage conditions, room temperature, -20°C, and -80°C respectively.

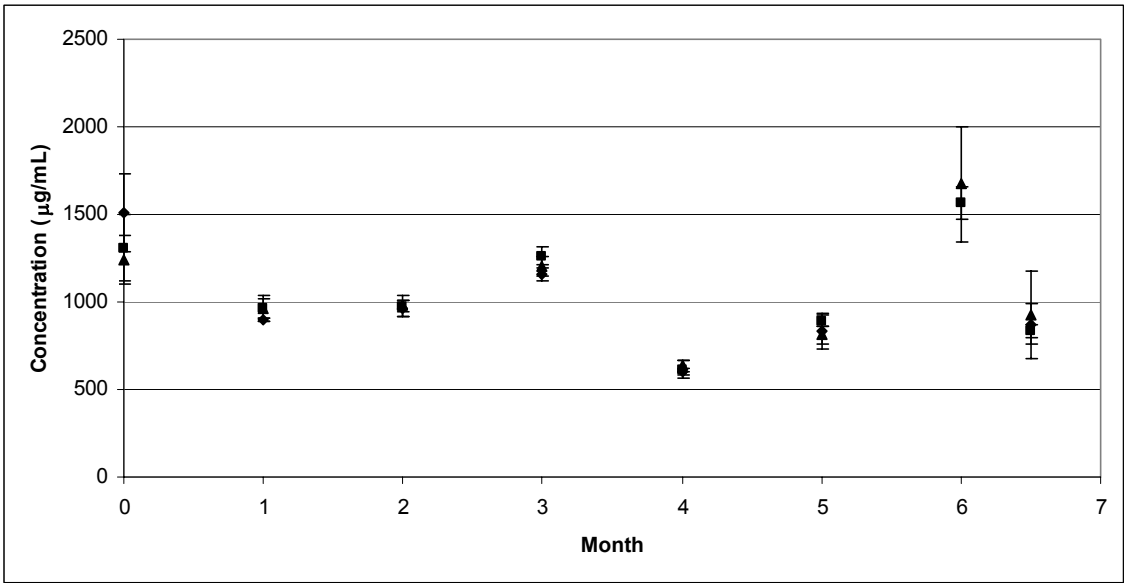


Figure 16: Concentration of spilanthol in 75% EtOH extracts of *S. acmella* stored for various lengths of time under various conditions. Data points represent the average of one injection of three replicate samples for each storage condition. The diamonds, squares, and triangles represent the three storage conditions, room temperature, -20°C, and -80°C respectively.

At the end of a six and a half month stability study, there appears to be no clear trend toward degradation in the concentration of spilanthol in the extracts at any

temperature. The closeness of all temperature replicates may indicate that the variation observed is most likely due to variations in instrument response over the time period evaluated rather than actual degradation in spilanthol. It should be noted that the data were all based on separate calibration curves run on the day of analysis. Absolute instrument response changes over time (due to a number of factors including fouling of the internal components of the instrument and drift in electronic components); therefore, in order to achieve acceptable intermediate precision, calibration curves should be included in the same run with the samples.

Identification of Alkylamides

The most abundant alkylamide identified in the ethanolic extract of *S. acmella* has a mass which corresponds to the protonated molecular ion of (2E,6Z,8E)-N-isobutyl-2,6,8-dectrienamide, or spilanthol, a known constituent of *S. acmella.*[11] The identity of the compound as spilanthol was confirmed by HPLC/ESI-MS-MS fragmentation, ^{1}H-NMR, and ^{13}C-NMR. The MS-MS fragments (obtained with collisionally activated dissociation in the ion trap) and UV absorption spectrum for spilanthol are shown in Figures 17 and 18, respectively. A λ_{max} value (wavelength of maximum absorbance) of 229 nm was observed, which is consistent the published value for spilanthol.[11]

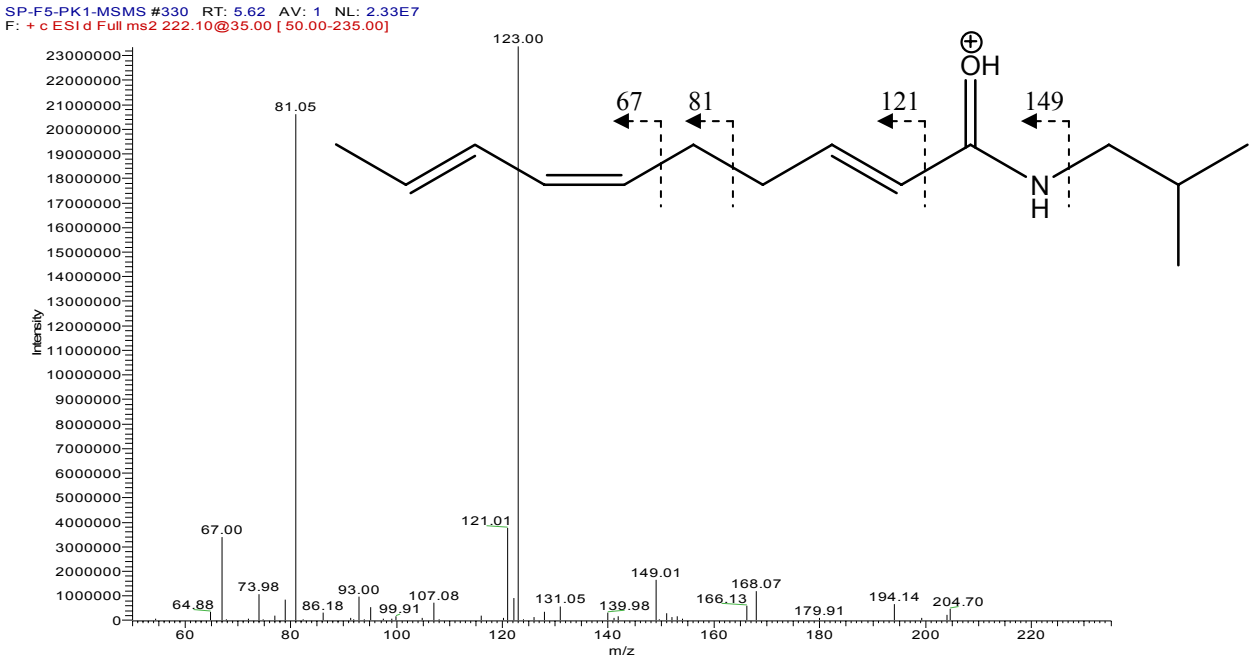


Figure 17: MS-MS spectrum obtained by activated dissociation with a collision energy of 35% of protonated molecular ion for spilanthol at m/z 222. The structure of protonated spilanthol with assignment of fragments is also shown.

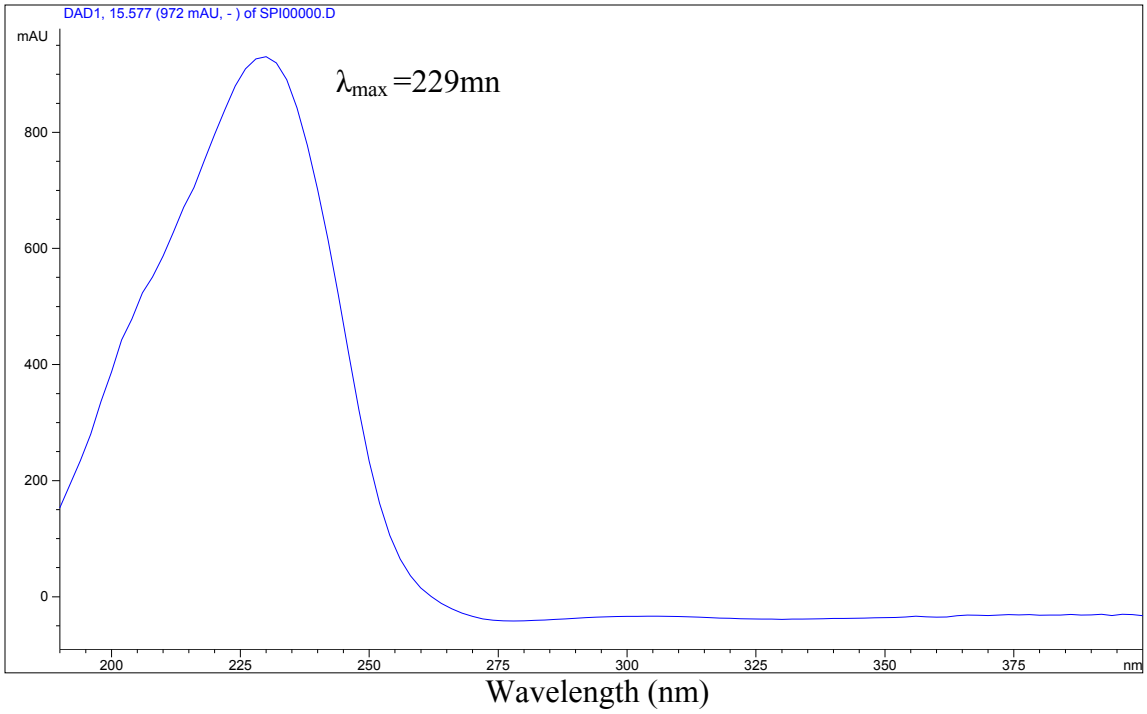


Figure 18: UV-VIS absorbance spectrum for spilanthol showing a λ_{max} of 229nm. This spectrum was obtained for the peak at 16.0 min. using the PDA detector of the HP1100 HPLC.

Ten additional alkylamides were tentatively identified in the ethanolic extract of *S. acmella* by comparison of molecular ion masses with molecular weights published for previously identified alkylamides. The selected ion chromatograms for the molecular ions of each of these compounds as well as spilanthol in the complex *Spilanthes acmella* extract are shown in Figure 19 and tentative structures can be seen in Figure 20.

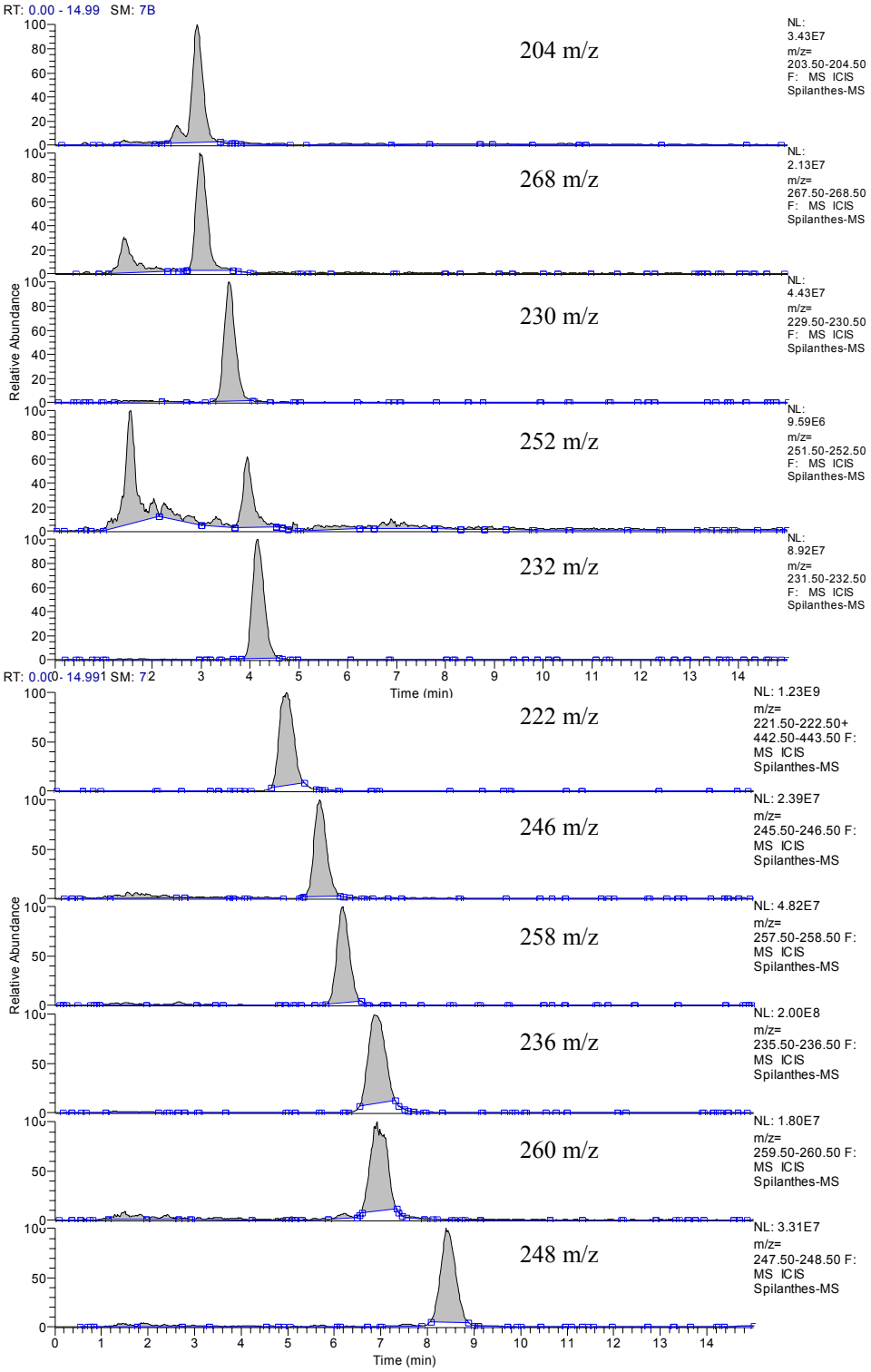


Figure 19: Selected ion chromatograms for molecular ions of tentatively identified alkylamides present in the complex *S. acmella* extract.

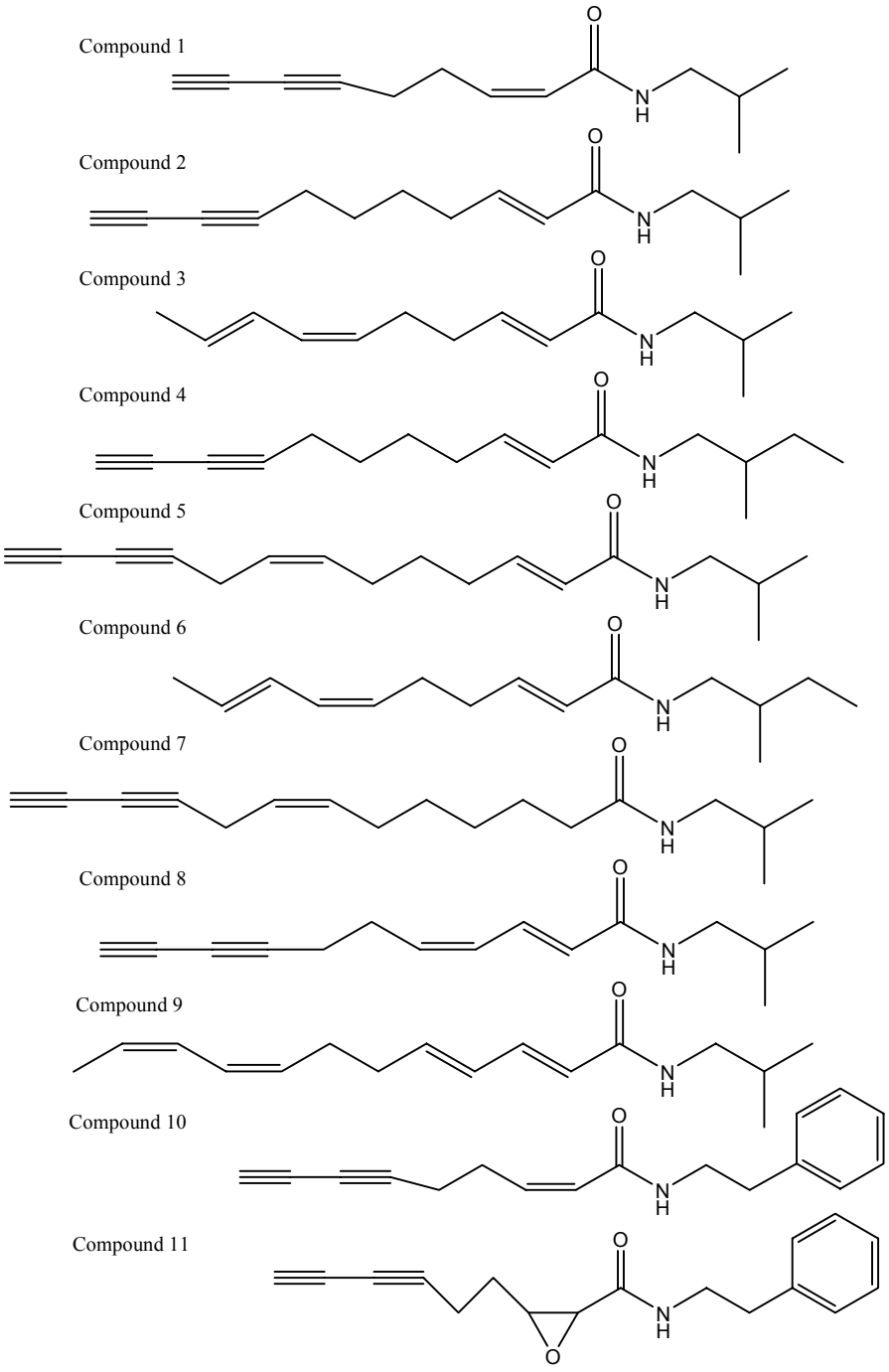


Figure 20: Structures of alkylamides tentatively identified in the ethanolic extract of *S. acmella*. Compound 3 was confirmed with NMR, compounds 1,2 and 4-9 were confirmed with MS-MS and compounds 10 and 11 were assigned based on molecular weight alone.

The structural assignments of the alkylamides seen above are listed in Table 2 and most are only tentative because, except in the case of compound 3 (spilanthol), they were not confirmed with NMR. However, comparisons of molecular ion masses were made with molecular weights published for previously identified alkylamides, whose structures had been eludicated based on NMR data in previous investigations.[11]

Table 2: Identification of alkylamides in an ethanolic extract of *S. acmella*.

#	Compound Name	MH^+ (m/z)	Retention Time (min)
1	(2Z)-N-isobutyl-2-nonene-6,8-diynamide	204.1	2.27
2	(2E)-N-isobutyl-2-undecene-8,10-diynamide	232.1	3.50
3	(2E,6Z,8E)-N-isobutyl-2,6,8-decatrienamide (spilanthol)	222.2	4.33
4	(2E)-N-(2-methylbutyl)-2-undecene-8,10-diynamide	246.1	5.04
5	(2E,7Z)-N-isobutyl-2,7-tridecadiene-10,12-diynamide	258.1	5.54
6	(2E,6Z,8E)-N-(2-methylbutyl)-2,6,8-decatrienamide	236.3	6.22
7	(7Z)-N-isobutyl-7-tridecene-10,12-diynamide	260.0	6.28
8	(2E,4Z)-N-isobutyl-2,4-undecadiene-8,10-diynamide	230.0	2.92
9	(2E,4E,8Z,10E)-N-isobutyl-dodeca-2,4,8,10-tetraenamide	248.1	7.76
10	(2Z)-N-phenethyl-2-nonene-6,8-diynamide*	252.2	3.36
11	N-phenethyl-2,3-epoxy-6,8-nonadiynamide*	268.0	2.35

*Compounds were only identified based on molecular ion values, MS-MS fragmentation patterns were not consistent with expected fragments.

Further validation of correct structural assignment was provided by MS-MS analysis. The MS-MS fragments (obtained with collisionally activated dissociation in the ion trap) for compounds 1-2 and 3-9 are shown the Figures 21-28. The structure of the protonated molecular ion and assignment of fragments is also shown. Alkylamides have previously been shown to fragment at both sides of the C-N bond[35] when subjected to

collisionally induced dissociation, and MS-MS spectra of nine alkylamides (compounds 1-9) showed fragments consistent with dissociation at these positions. The molecular ion masses of compounds 1-7 and 10-11 matched published molecular weight of nine alkylamides that have previously been confirmed as components of *S. acmella*.[3, 11] The molecular ion of compound 8 matched the molecular weight of the alkylamide (2E,4Z)-N-isobutyl-2,4-undecadiene-8,10-diynamide, that has been reported in *S. ciliata*[22, 23] and *S. alba,*[19] although no reports of this compound in *S. acmella* have been published thus far. Compound 9 has a molecular ion that matches published data for (2E,4E,8Z,10E)-N-isobutyl-dodeca-2,4,8,10-tetraenamide found in *S. mauritiana,*[21] *S. oppositifolia*,[26] *S. ciliate*,[22, 23] and *S. alba,*[19] but not in *S. acmella*. Both (2E,4Z)-N-isobutyl-2,4-undecadiene-8,10-diynamide (tentative identification of compound 8) and (2E,4E,8Z,10E)-N-isobutyl-dodeca-2,4,8,10-tetraenamide (tentative identification of compound 9) have previously been identified in *Echinacea purpurea*. The MS-MS spectra of compounds 8 and 9 match published MS-MS data for (2E,4Z)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide and (2E,4E,8Z,10E)-*N*-isobutyl-dodeca-2,4,8,10-tetraenamide, respectively, in *Echinacea purpurea.*[35] Compounds 10 and 11 gave only very small signals in the base peak chromatogram and their MS-MS fragmentation patterns did not show the expected fragments that would be produced by dissociation of the C-N bonds within the molecule. The discrepancy of the MS-MS fragmentation may be due to mis-identification of the molecular ion. It is also likely that the presence of the phenyl group instead of the isobutyl or 2-methylbutyl group attached to the amide

nitrogen results in a different fragmentation pattern than has been observed previously for 2-methylbutyl and isobutylamides.

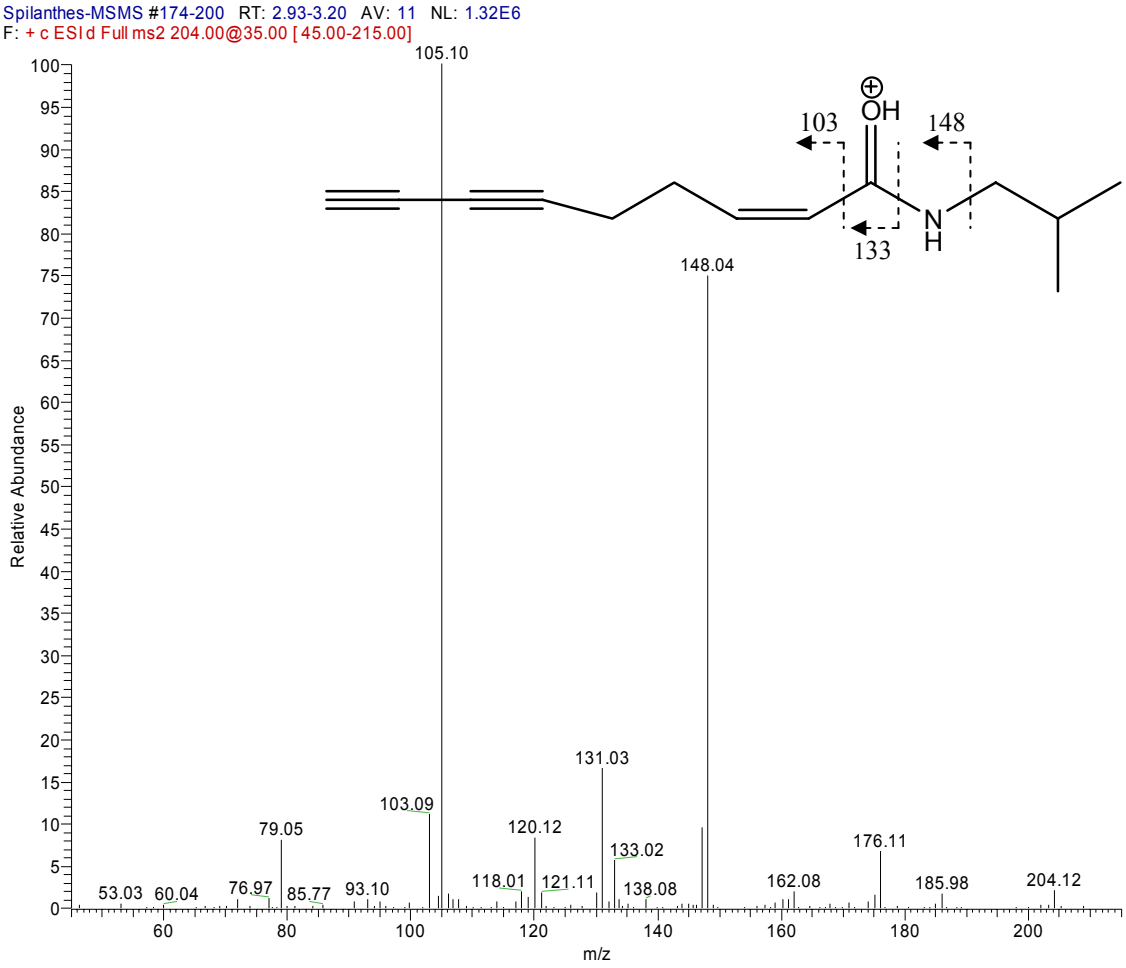


Figure 21: MS-MS spectrum obtained by activated dissociation with a collision energy of 35% of protonated molecular ion for (2Z)-*N*-isobutyl-2-nonene-6,8-diynamide at m/z 204.

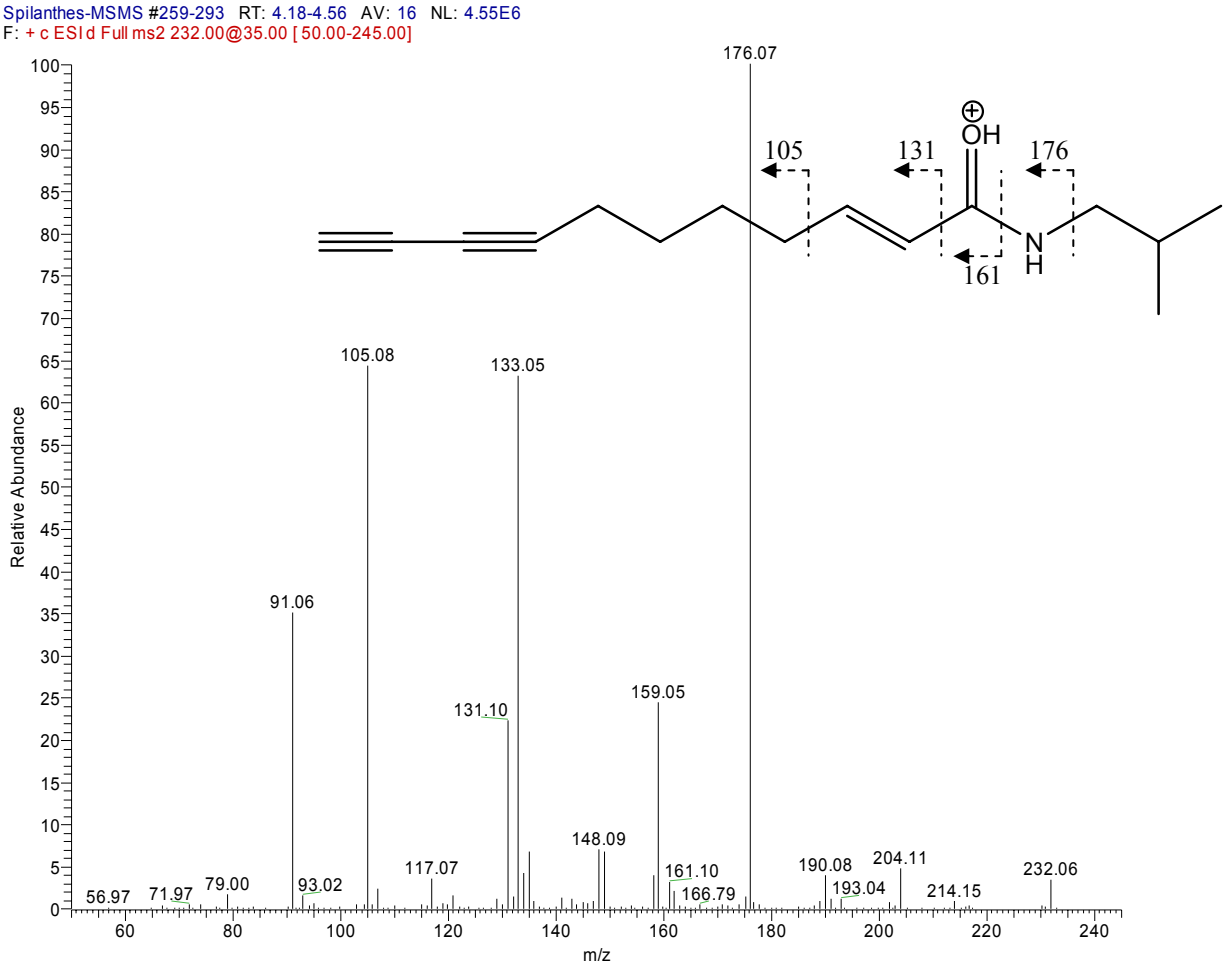


Figure 22: MS-MS spectrum obtained by activated dissociation with a collision energy of 35% of protonated molecular ion for (2E)-*N*-isobutyl-2-undecene-8,10-diynamide at m/z 232.

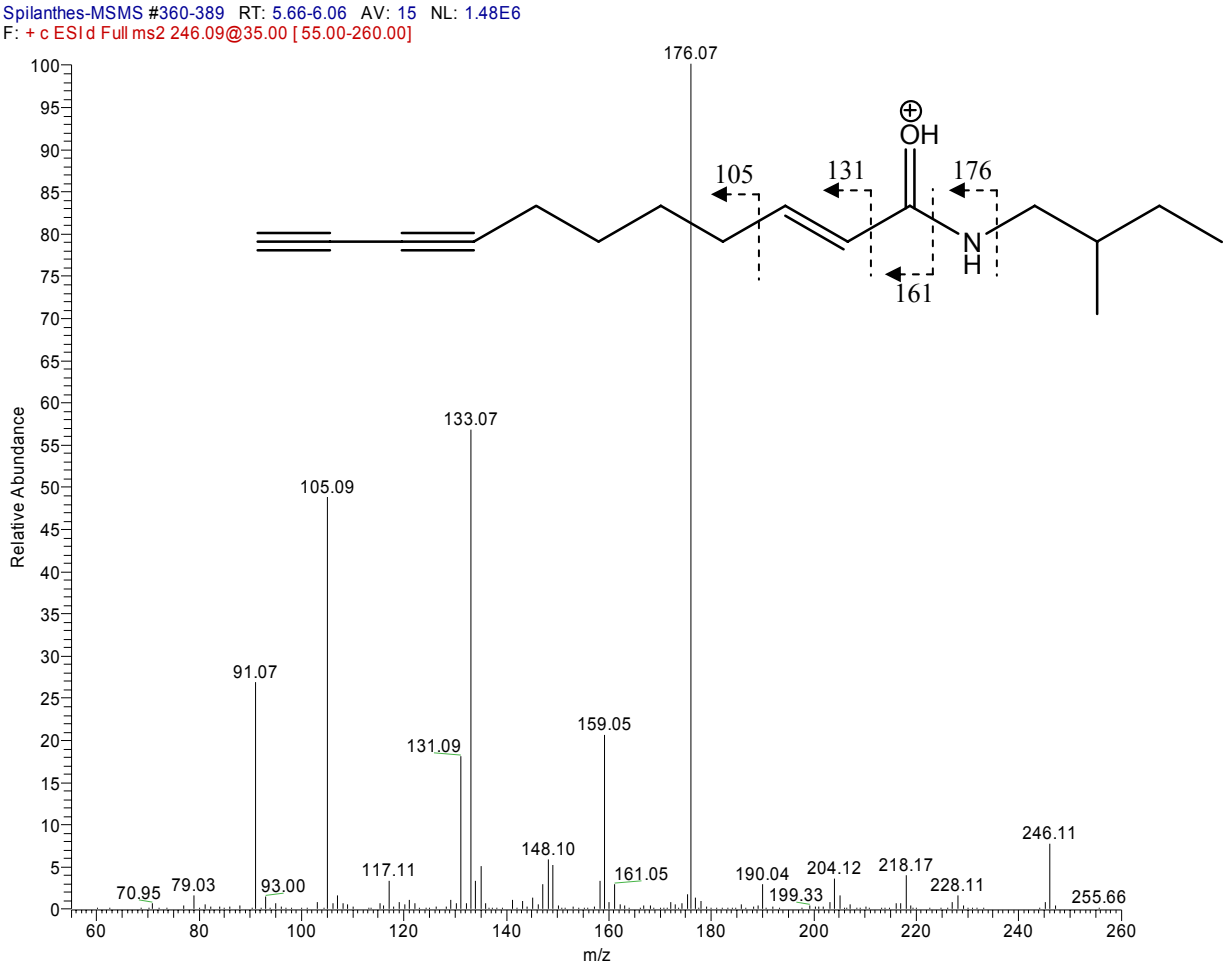


Figure 23: MS-MS spectrum obtained by activated dissociation with a collision energy of 35% of protonated molecular ion for (2E)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide at m/z 246.

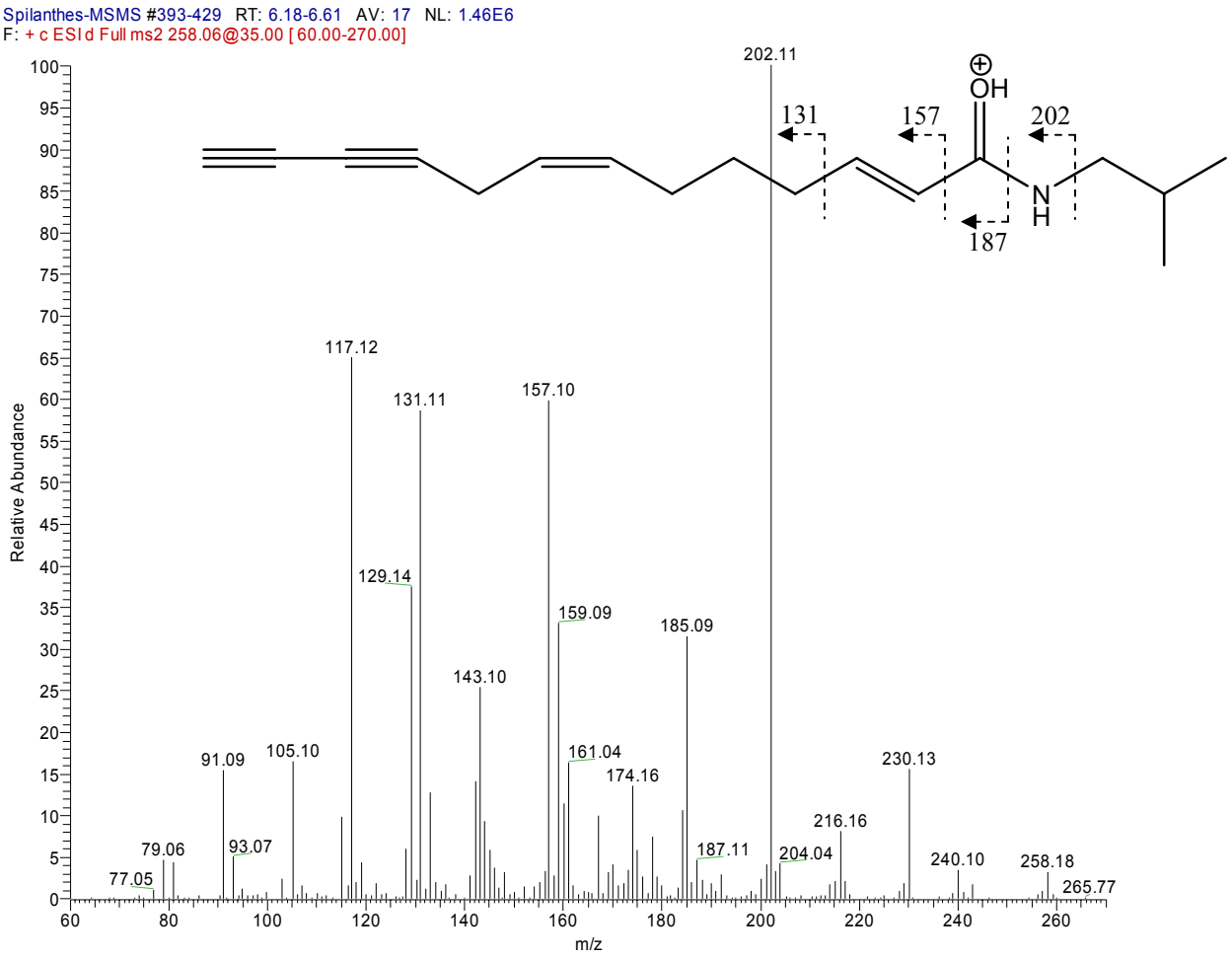


Figure 24: MS-MS spectrum obtained by activated dissociation with a collision energy of 35% of protonated molecular ion for (2E,7Z)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide at m/z 258.

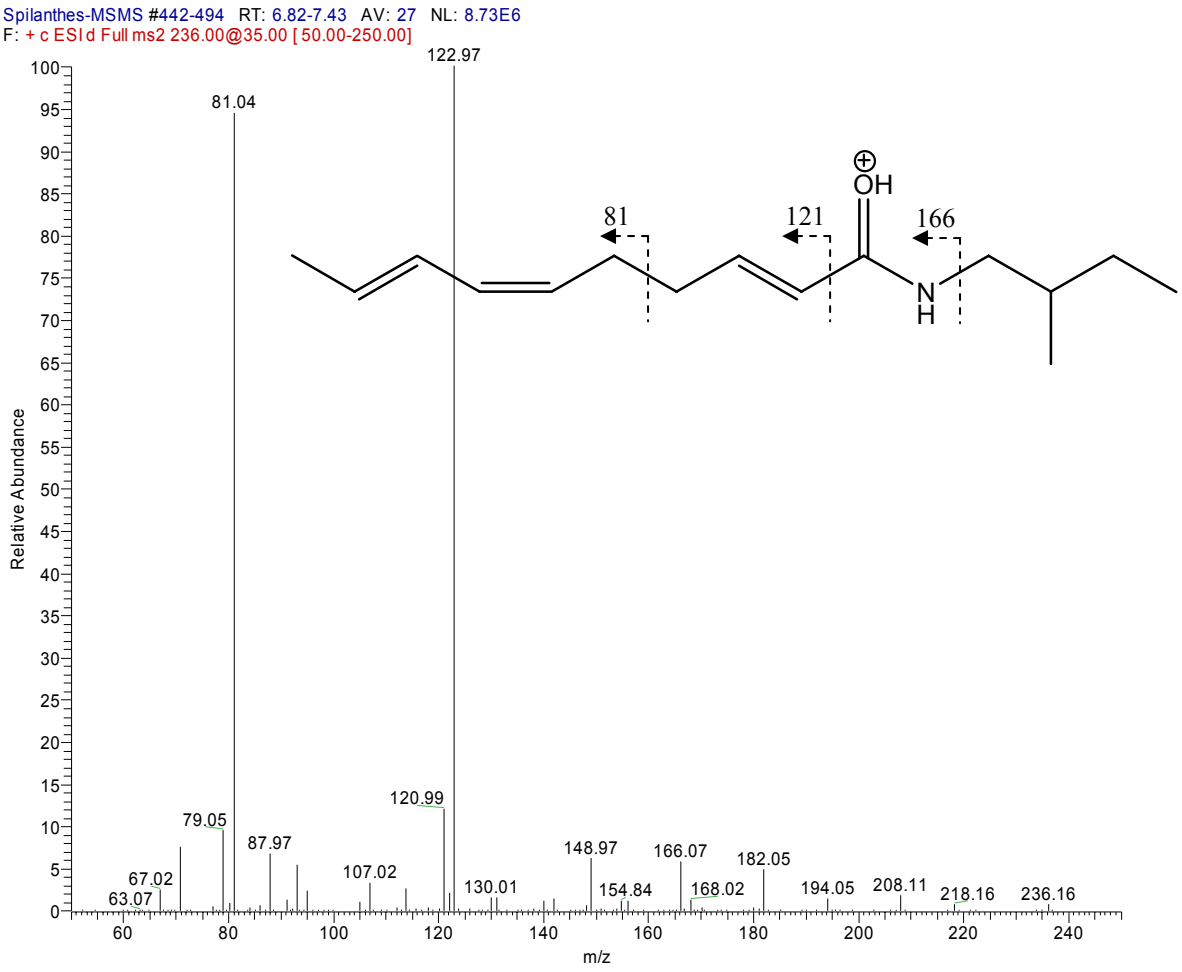


Figure 25: MS-MS spectrum obtained by activated dissociation with a collision energy of 35% of protonated molecular ion for (2E,6Z,8E)-*N*-(2-methylbutyl)-2,6,8-decatrienamide at m/z 236.

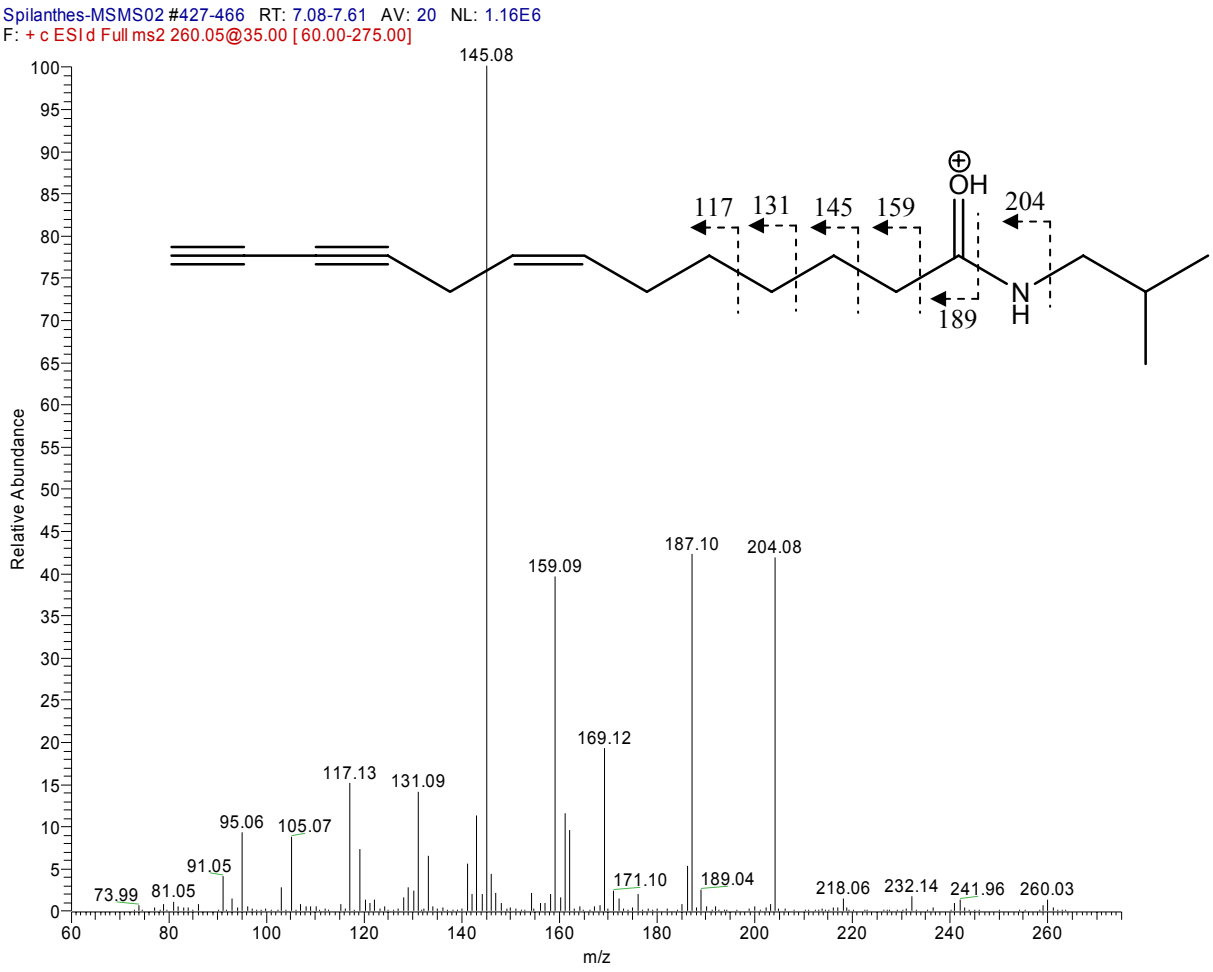


Figure 26: MS-MS spectrum obtained by activated dissociation with a collision energy of 35% of protonated molecular ion for (7Z)-*N*-isobutyl-7-tridecene-10,12-diynamide at m/z 260.

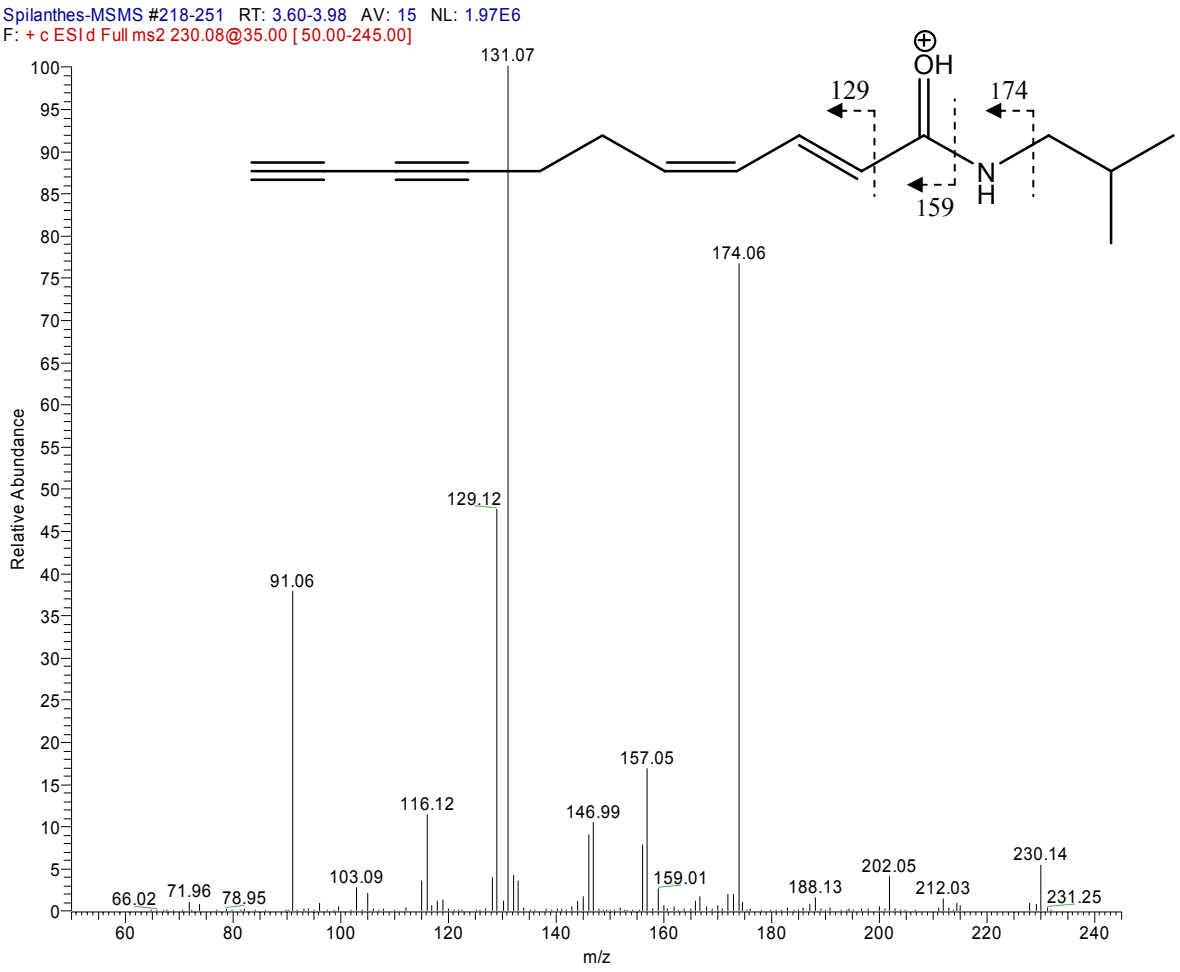


Figure 27: MS-MS spectrum obtained by activated dissociation with a collision energy of 35%of protonated molecular ion for (2E,4Z)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide at m/z 230.

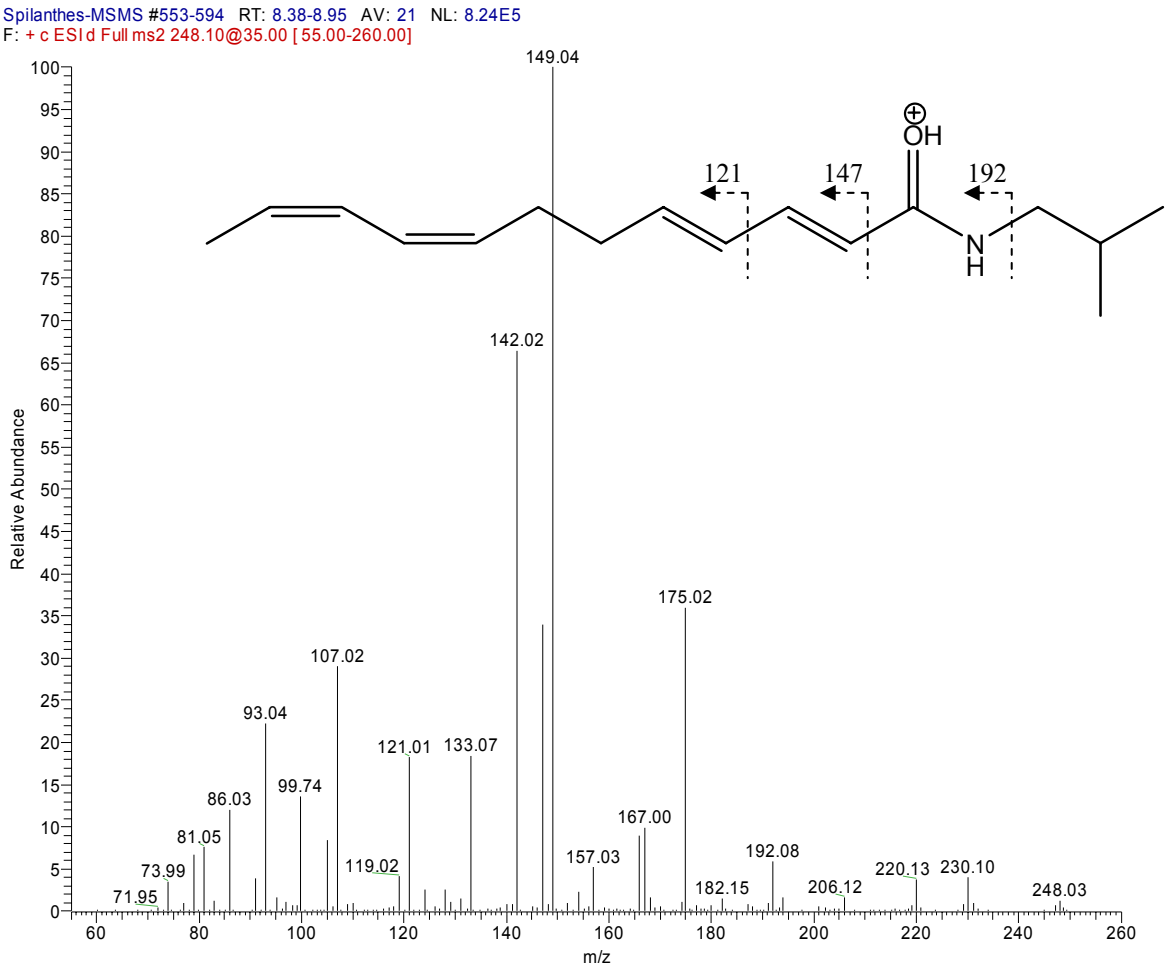


Figure 28: MS-MS spectrum obtained by activated dissociation with a collision energy of 35%of protonated molecular ion for (2E,4E,8Z,10E)-*N*-isobutyl-dodeca-2,4,8,10-tetraenamide at m/z 248.

The stereochemistry of spilanthol was confirmed using ^{1}H-NMR and ^{13}C-NMR. The shifts observed for the isolated spilanthol with ^{1}H-NMR and ^{13}C NMR matched previously published values [11] for spilanthol and are as follows: ^{1}H-NMR ($CDCl_3$, 500 MHz), δ 6.82 (dt, 1H, J=15Hz), 6.28 (br dd, 1H, J=15Hz), 5.97 (dd, 1H, J=11Hz), 5.77 (br d, 1H, J=15Hz), 5.69 (dq, 1H, J=15Hz), 5.26 (dt, 1H, J=11Hz), 3.14 (dd, 2H), 2.29

(m, 4H), 1.77 (m, 1H), 1.77 (d, 3H), 0.92 (d, 6H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.06, 143.78, 130.10, 129.52, 127.79, 126.77, 124.13, 46.92, 32.19, 28.68, 26.44, 20.22, 18.44. The ^{1}H-NMR and ^{13}C NMR spectra can be seen in Figures 29 - 34.

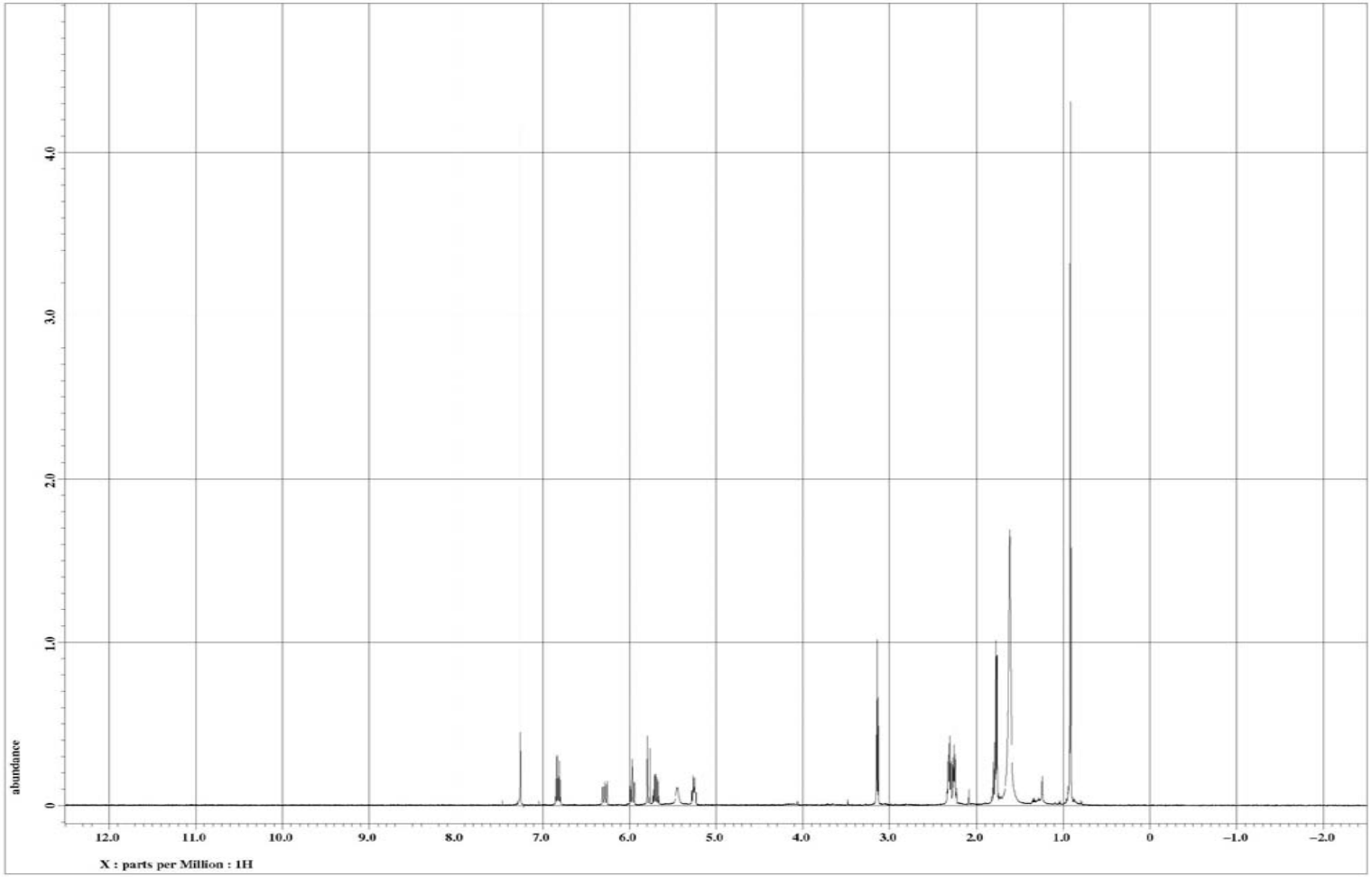


Figure 29: ^{1}H-NMR spectra for spilanthol ($CDCl_3$, 500 MHz).

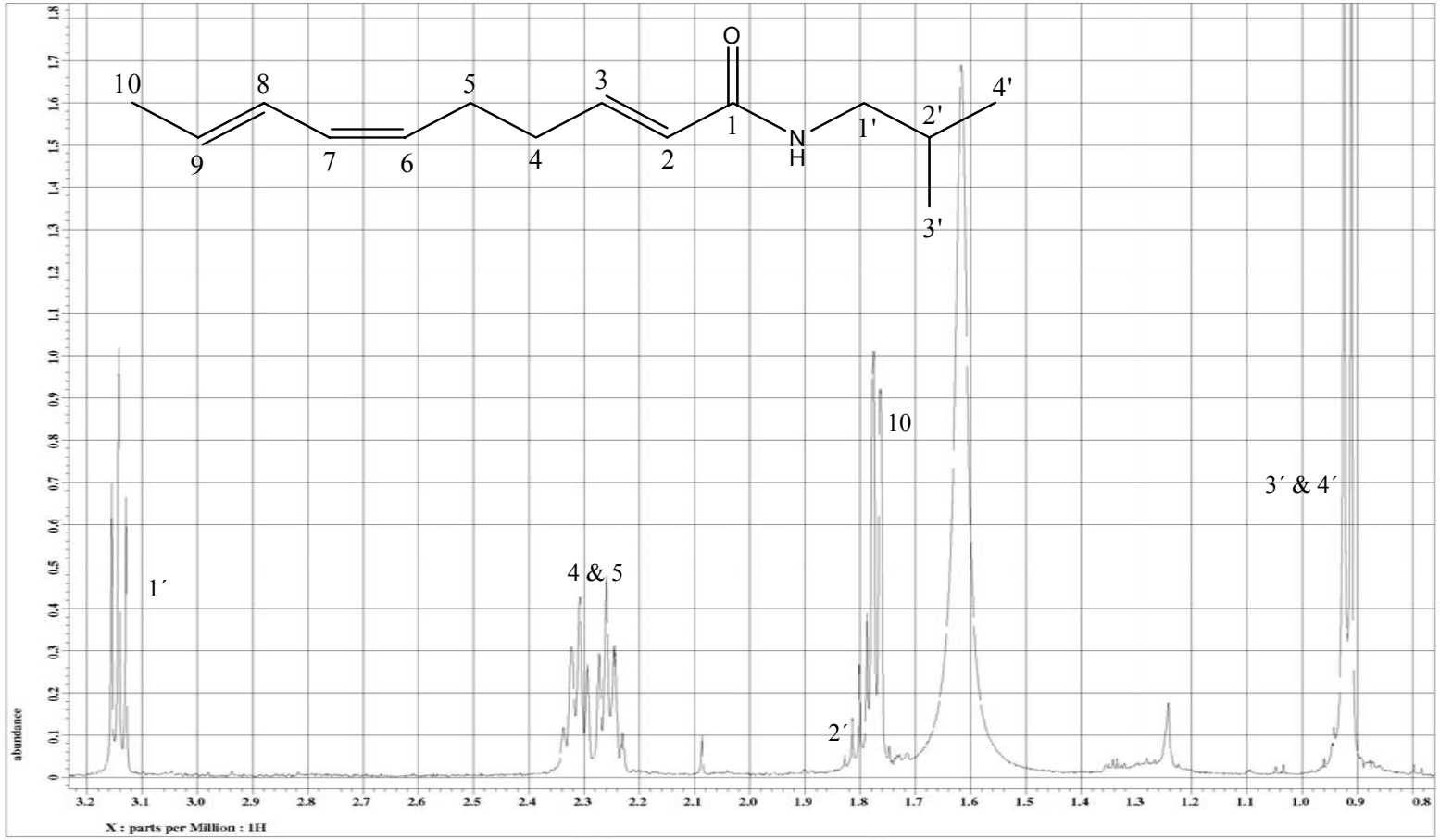


Figure 30: ^{1}H-NMR of spilanthol zoomed in from 0.8-3.2 ppm ($CDCl_3$, 500 MHz).

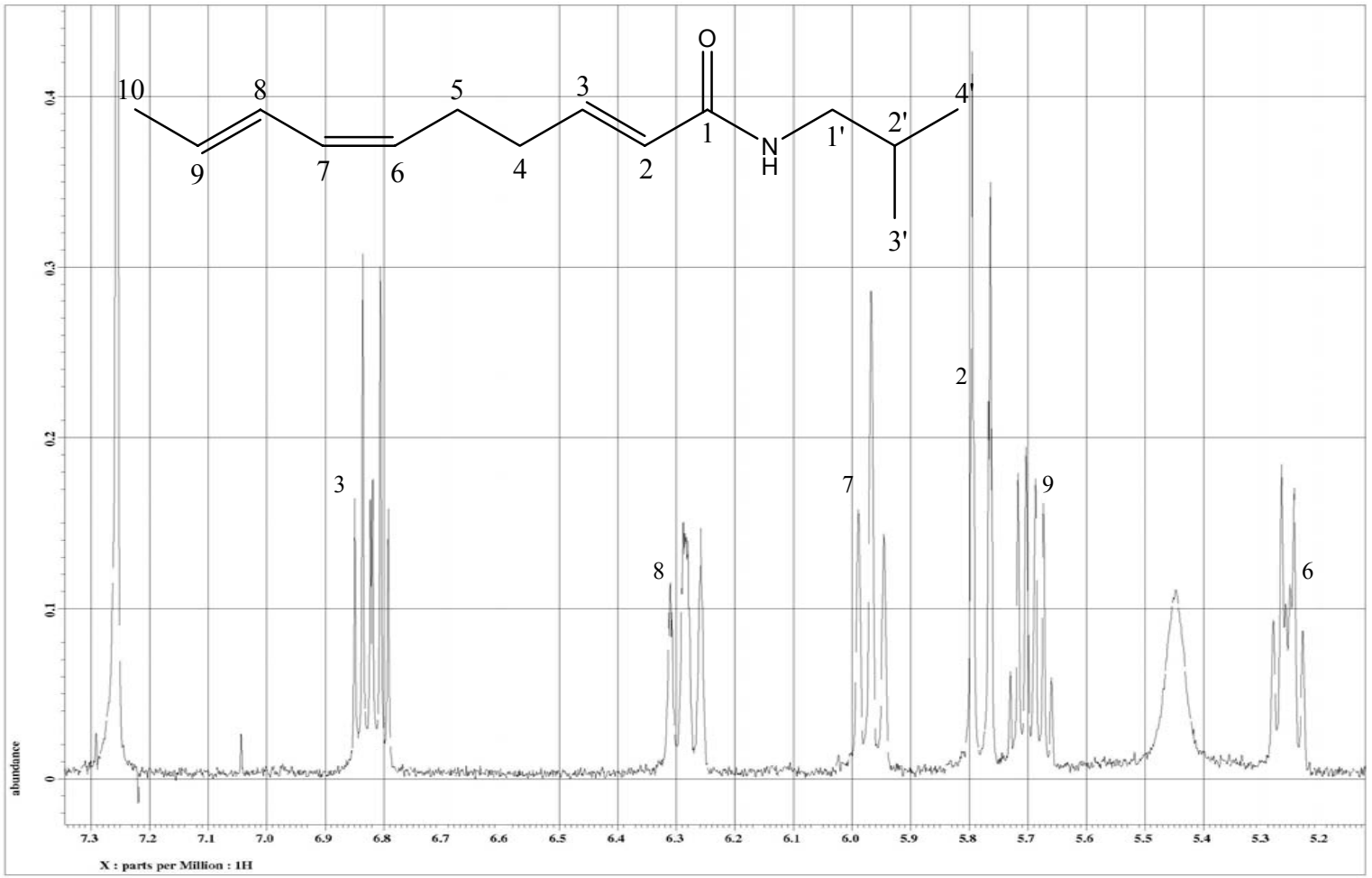


Figure 31: ^{1}H-NMR of spilanthol zoomed in from 5.2-7.3 ppm ($CDCl_3$, 500 MHz).

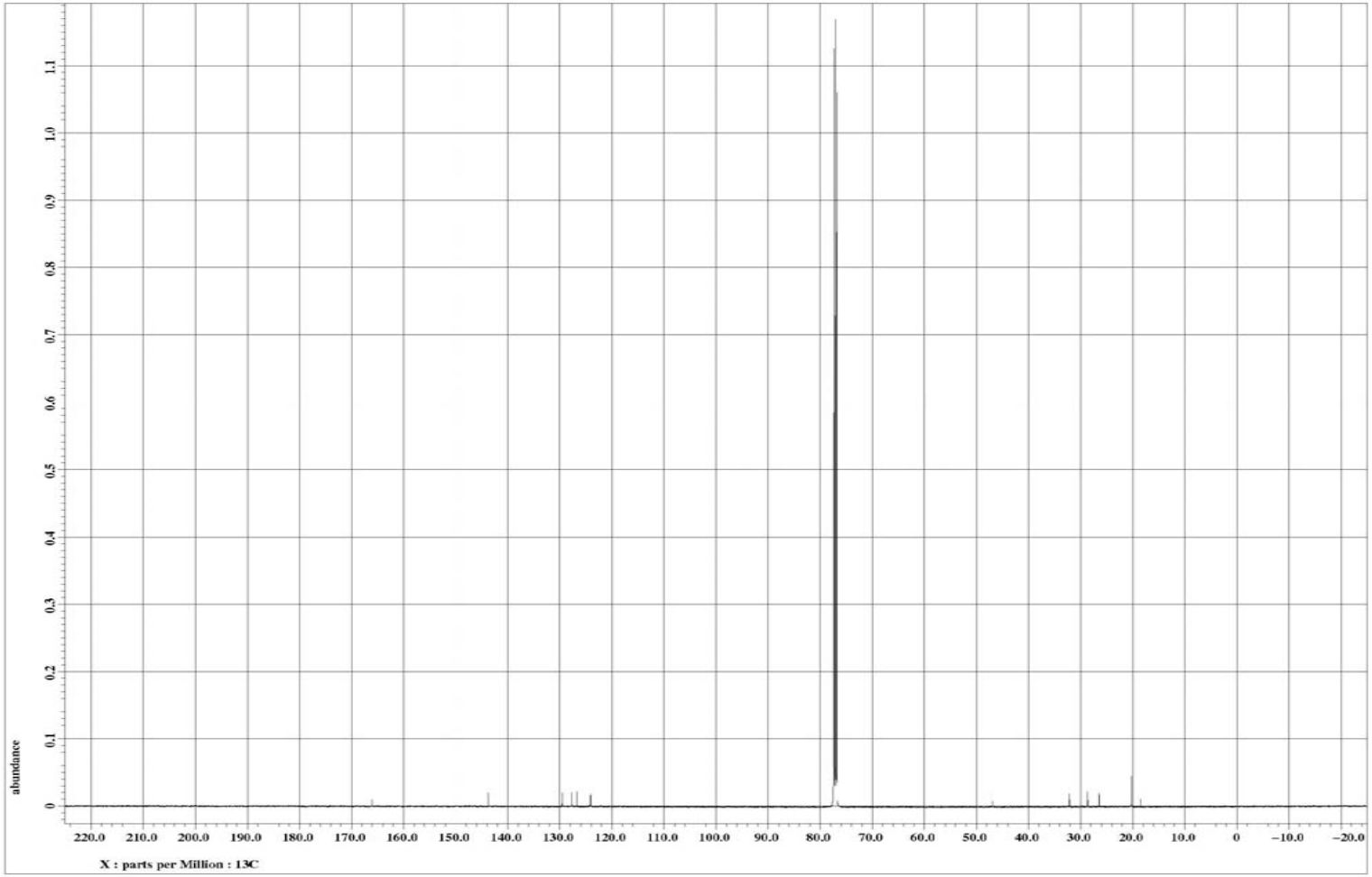


Figure 32: ^{13}C-NMR for spilanthol ($CDCl_3$, 125 MHz).

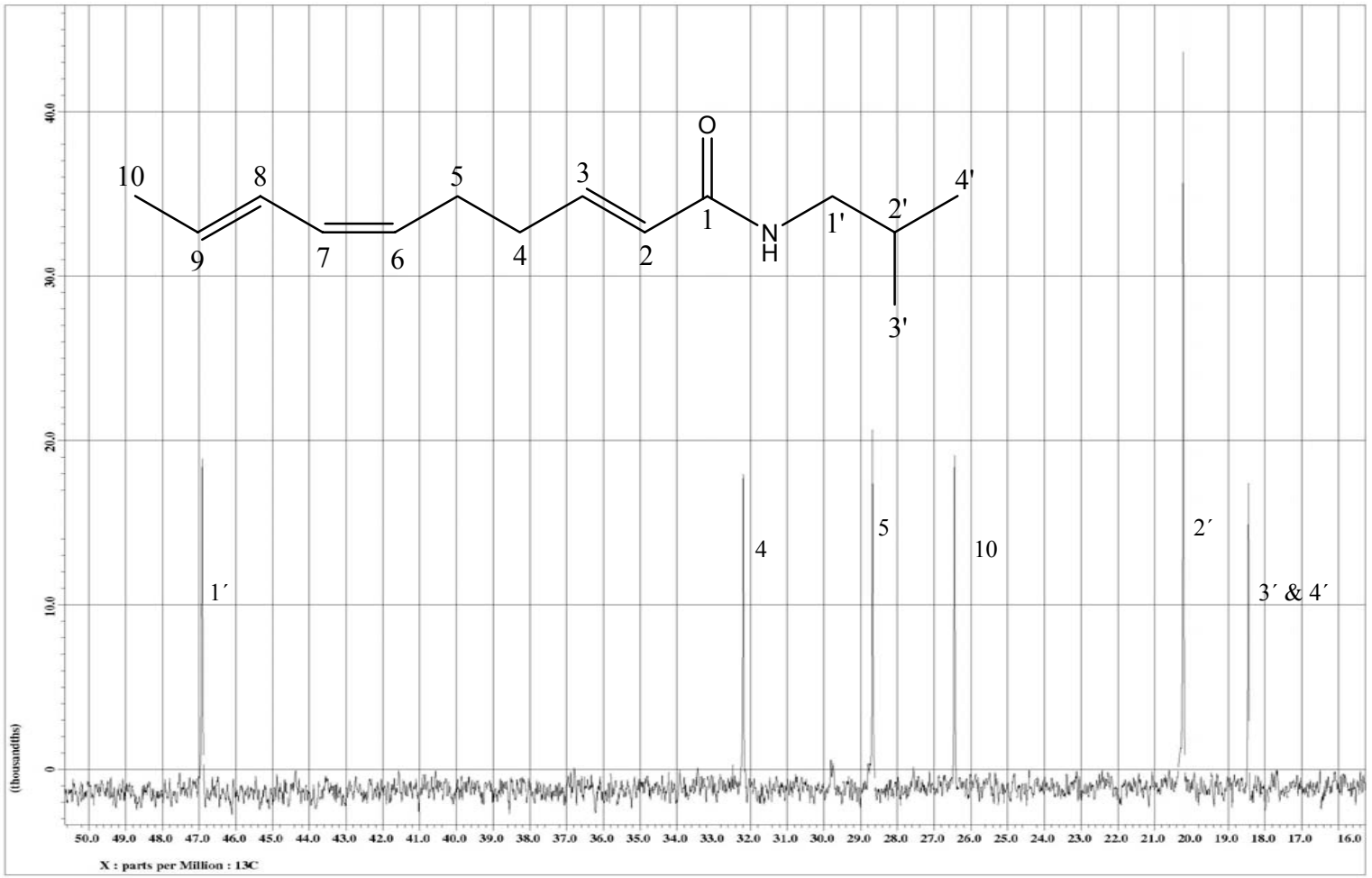


Figure 33: ^{13}C-NMR for spilanthol zoomed in from 16-50 ppm ($CDCl_3$, 125 MHz).

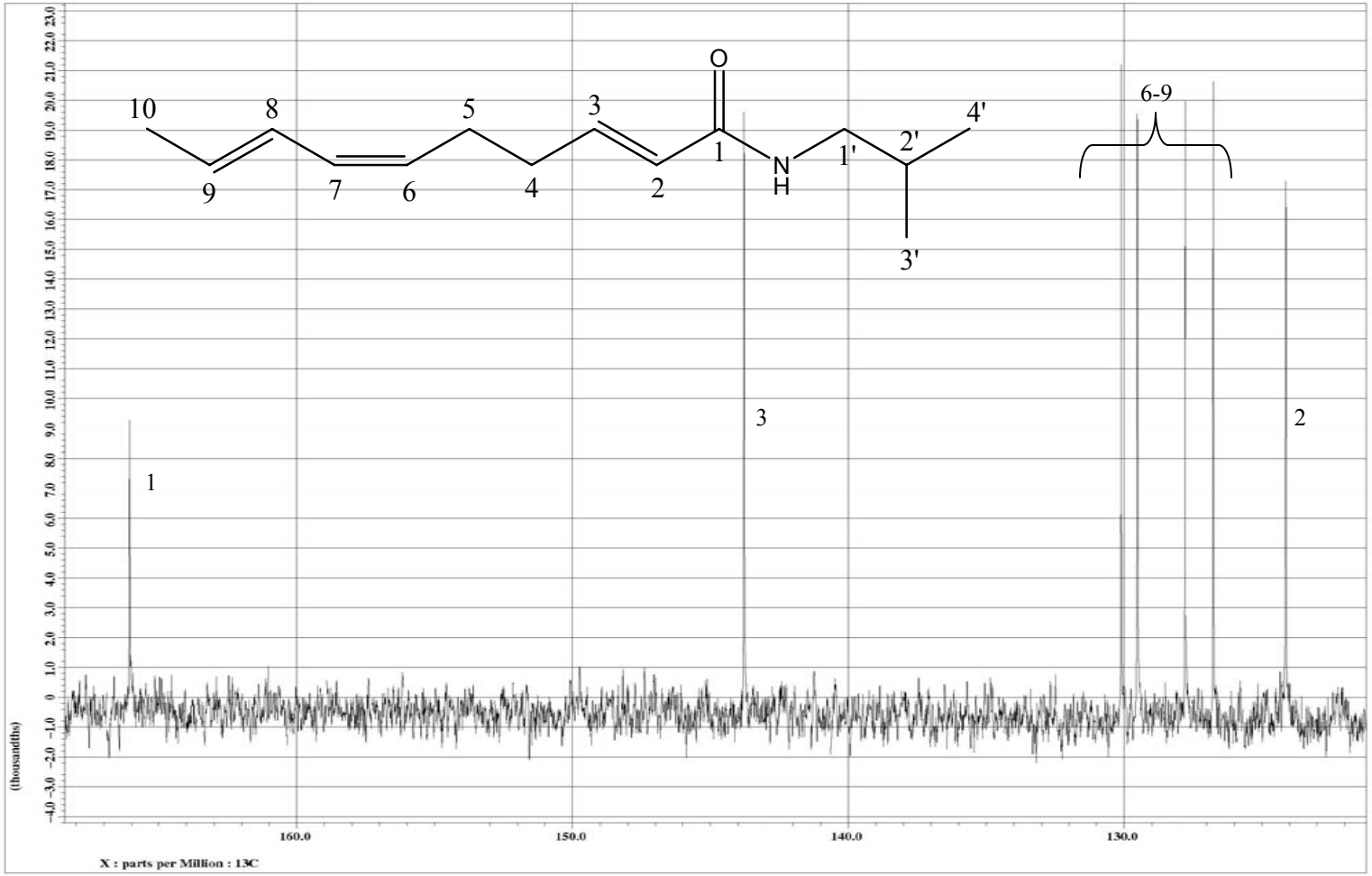


Figure 34: ^{13}C-NMR for spilanthol zoomed in from 122-166 ppm ($CDCl_3$, 125 MHz).

Method Validation

The stock solution of spilanthol (isolated from *S. acmella* as described previously) was serially diluted to make five standards at concentrations of 1.4 μM, 4.5 μM, 14 μM, 45 μM, and 140 μM. The calibration standards were analyzed using HPLC/ESI-MS as described earlier. A calibration was generated by plotting the log of the sum of the peak areas of the selected ion chromatograms for the spilanthol ions versus the log of the concentration. Because spilanthol forms a dimer in the gas phase, the peak area for m/z 443 (proton bound dimer) was doubled and added to the peak area of m/z 222 (protonated molecular ion) to account for all of the spilanthol response. Figure 35 shows a calibration curve for spilanthol. The correlation coefficient, y-intercept, slope of regression line and plot were evaluated and the results can be seen in Table 3. LOD and LOQ were determined based on signal to noise ratio of 3:1 and 10:1 respectively and are also presented in Table 3.

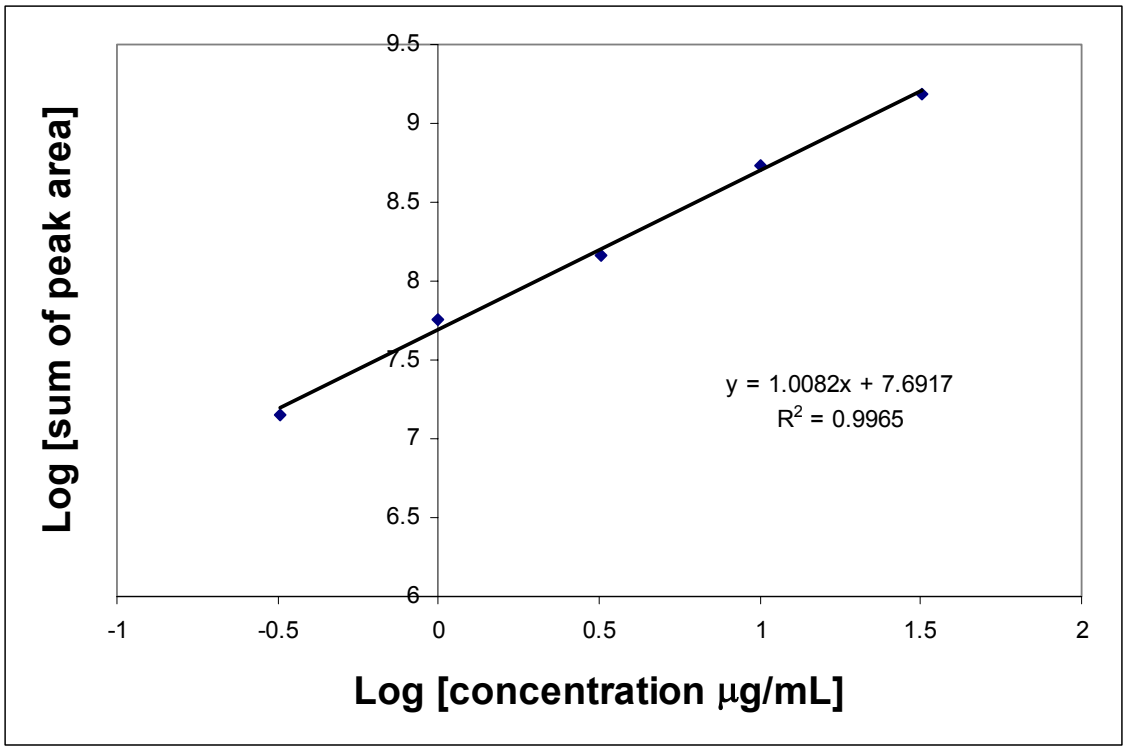


Figure 35: Calibration curve for spilanthol.

Table 3: Regression parameters, detection limit, and quantification limit. These calculations were based upon triplicate analyses of five standard solutions of spilanthol. The MDL and MQL were determined based on signal-to-noise ratios of 3:1 and 10:1 respectively, with the noise determined from injection of a solvent blank (95% ethanol.)

Validation Parameter	**Result**
Correlation Coefficient	0.9965
y-intercept	7.69 ± 0.02
Slope of regression line	1.01 ± 0.02
Number of data points	15
Range	0.45μM to 450 μM
Limit of Detection	0.27 μM
Limit of Quantitation	0.45 μM

Figure 36 shows representative selected ion chromatograms used in determining LOD and LOQ. Due to poor synchronization between the mass spectrometer and the HPLC, the retention times do not line up in the figure.

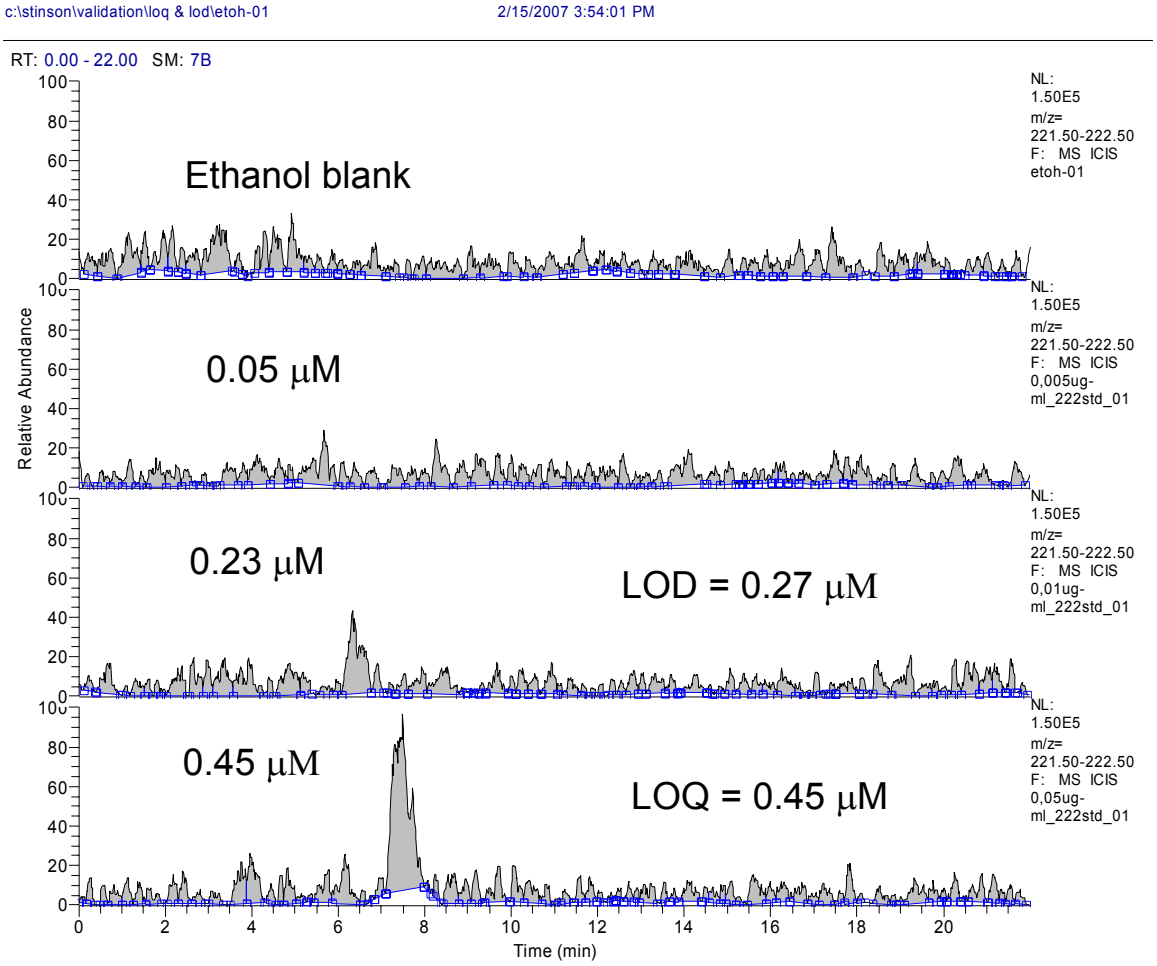


Figure 36: Selected ion chromatograms of spilanthol standards used to determine LOQ and LOD.

The results of the studies that were conducted to evaluate precision are shown in Table 4. The %RSD values for both repeatability and intermediate precision were less than 6%, showing excellent reproducibility of the method. Precision data was obtained from analyzing five standards in triplicate over three days. The repeatability was determined within a day and intermediate precision was calculated using the average back calculated concentration per day and determining the % relative standard deviation. The % residuals were determined form the intermediate precision average of each concentration.

Table 4: Precision Data

Theoretical Concentration (µg/mL)	Measured Concentration (µg/mL)	Residuals (%)	Repeatability (%)	Intermediate Precision (%)
0.3	0.3	-8%	6%	2%
1.0	1.1	7%	4%	2%
3.2	3.3	5%	2%	1%
10.0	9.8	-2%	1%	1%
31.6	30.7	-3%	6%	1%

Antibacterial Assays

The antibacterial activity was tested by the agar diffusion method with top agar. A sample that shows antibacterial activity will inhibit the growth of bacteria around the disk containing the sample. The size of the inhibition zone is not directly indicative of the antibacterial strength of the compound due to several reasons: not all compounds will have the same rate of diffusion through the agar, the plates do not have exactly the same

concentration of bacteria, and the disks containing the sample may not have the same concentrations. Figure 37 shows kanamycin (30µg), a known antibiotic, used as the positive control in these experiments. There is a distinct zone of inhibition around the disk containing the antibiotic which indicates the absence or inhibition of growth of bacteria. The spilanthol, neat Spilanthes extract, and 10x concentrated extract tests showed no zone of inhibition and, hence, no antibacterial activity at that concentration against *Staphylococcus aureus*. The same samples were also tested with 5mg/mL berberine and showed no additional inhibition beyond the berberine control. All tests were done in triplicate and Figures 38 and 39 show one replicate of the assay for each sample tested.

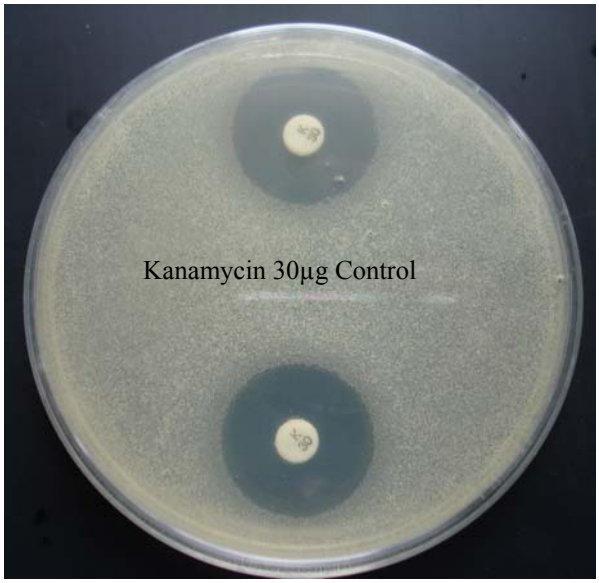


Figure 37: Antibacterial assay results for 30µg kanamycin antibiotic disks in duplicate.

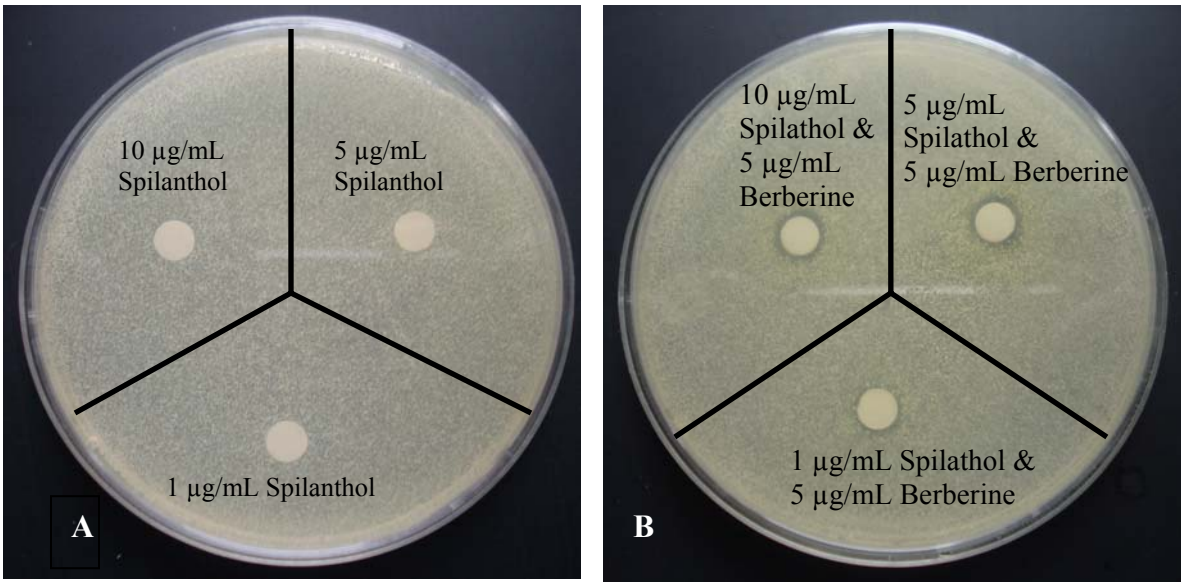


Figure 38: Antibacterial assay results for spilanthol a) without berberine b) with berberine.

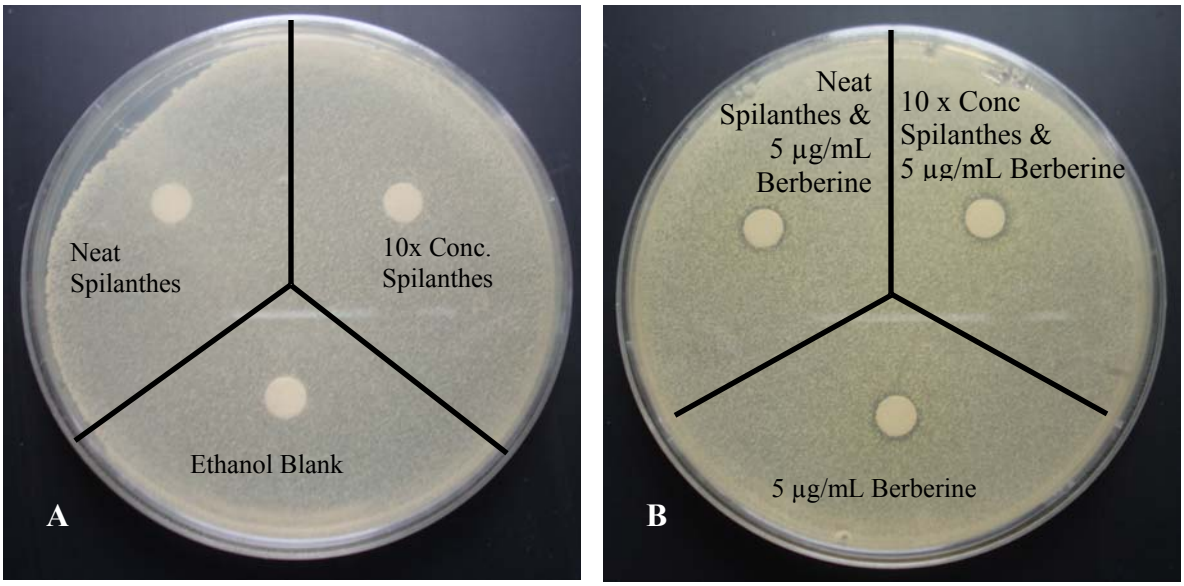


Figure 39: Antibacterial assay results for Spilanthes extract a) without berberine b) with berberine.

CHAPTER IV

CONCLUSIONS

Summary and Significance

A rapid and simple reversed-phase HPLC/ESI-MS method was developed and validated according to ICH guidelines for the simultaneous identification and quantification of spilanthol in ethanolic extracts of *S. acmella*. This is the first example of the use of electrospray ionization rather than electron impact ionization for determination of *Spilanthes* alkylamides. The use of this soft ionization technique coupled to a mass spectrometer with an ion trap mass analyzer enables the identification of the molecular ion and the collection of MS-MS spectra. Our studies demonstrate that such MS-MS spectra can be very useful for identification of alkylamides in complex *Spilanthes* extracts.

The ability to interface directly to HPLC separations is another advantage of this new method analysis. On-line HPLC-MS analysis facilitates the rapid determination of alkylamide content in the same ethanolic extracts of *Spilanthes* that are typically used by consumers. To date, there have been no published methods for quantification of alkylamides in ethanolic *Spilanthes* extracts. By demonstrating an approach to quantitatively determine spilanthol concentration in *Spilanthes* extracts, the method

published here will facilitate further assessment of the biological benefits and risks of therapeutic use of *Spilanthes*.

The method developed during this research enabled tentative identification (based on molecular weight and fragmentation patterns) of six alkylamides previously identified in *S. acmella* and two additional alkylamides not previously identified in *S. acmella*. The alkylamides tentatively identified in *S. acmella* were as follows: (2Z)-*N*-isobutyl-2-nonene-6,8-diynamide, (2E)-*N*-isobutyl-2-undecene-8,10-diynamide, (2E)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, (2E,7Z)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide, (2E,6Z,8E)-*N*-(2-methylbutyl)-2,6,8-decatrienamide, (7Z)-*N*-isobutyl-7-tridecene-10,12-diynamide, (2E,4Z)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide, (2E,4E,8Z,10E)-*N*-isobutyl-dodeca-2,4,8,10-tetraenamide. The structure of the most abundant of these alkylamides, (2E,6Z,8E)-*N*-isobutyl-2,6,8-decatrienamide (spilanthol), was confirmed by NMR.

The investigations published here also demonstrate for the first time the stability of spilanthol in an ethanolic extract. For the 75% ethanolic extract of fresh whole plants of *S. acmella*, there was no significant degradation of spilanthol (at least none detectable beyond the scatter of the data) during storage over six and a half months at room temperature, -20°C, and -80°C. These results indicate that alkylamide constituents of *S. acmella* extracts can be tested for biological activity over a time period of at least six months without concern about major degradation of alkylamides.

Complex *S. acmella* extract and isolated spilanthol showed no antibacterial activity against *Staphylococcus aureus;* nor did they show activity in combination with berberine.

Thus, spilanthol appears not to be useful against *Staphylococcus aureus* or as a synergist to enhance the antibacterial activity of berberine against this bacterium. These results are consistent with literature reports which report no activity against *Staphylococcus aureus*. Another study investigated *Spilanthes mauritiana* and reported that it demonstrates very low antibacterial activity against 105 strains of bacteria. *Spilanthes* is traditionally used to treat mouth sores and sore throats; this may be due to the analgesic action of the alkylamide constituents rather than any antibacterial activity.

Future Research

To broaden the scope of the method published here, isolation and positive identification of more alkylamides would be useful. Once the alkylamides are isolated, NMR analysis can confirm the identification that has been based so far solely on mass spectrometric data. The isolated alkylamides can then be used to create standards and facilitate quantification of additional constituents in *Spilanthes*.

Another aspect that can be investigated in addition to this research is structure of the alkylamides tentatively identified as (2Z)-*N*-phenethyl-2-nonene-6,8-diynamide and *N*-phenethyl-2,3-epoxy-6,8-nonadiynamide. Their MS-MS fragmentation patterns did not show the expected fragments that would be produced by dissociation of the C-N bonds within the molecule which may be due to mis-identification of the molecular ion or the presence of the phenyl group instead of the isobutyl or 2-methylbutly group attached to the amide nitrogen. By comparing NMR data and MS-MS fragmentation patterns, the identity of these two alkylamides could be discerned.

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